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Tesi di Dottorato

**“New Insights In Veterinary Forensic Medicine and
Pathology”**

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For those who cannot speak for themselves,

*For my Dad...
How ironic is it that
I wasted all these years not listening to you.
But now that you're not here, I'm living life exactly
how you told me to*

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List of abbreviations

PMI	Post Mortem Interval
IHC	Immunohistochemistry
ASA	Animal Sexual Abuse
DSLR	Digital single-lens reflex
ICC	Intraclass Correlation Coefficient
HE	Hematoxylin and Eosin
SUID	Sudden and Unexpected infant death
SIDS	Sudden infancy death Syndrom
CPV-2	Canine Parvovirus Type 2
PCR	Polymerase Chain Reaction
PMR	Proportional Mortality Ratio

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Over the last years, the knowledge on the veterinary forensic medicine and pathology has experienced a rapid increase as evidenced by number of peer-reviewed publications, textbooks and inclusion of the topic in many veterinary medical conferences. However, most of the information in veterinary forensic medicine is still acquired by human forensic literature. This lack of information is currently considered a serious problem in veterinary forensic medicine. Indeed, although it is undeniably true that the mechanisms of forensic injuries as well as the post-mortem cadaveric changes are similar between humans and animals, the different morphology, weight and tissue resistance of animals compared to human anatomy and other species-specific factors make the information validated in human forensic medicine not always applicable in the veterinary forensic field. In addition, in human forensic medicine, the macroscopic examination associated with the histological analysis is often not sufficient to determine the victim's cause and manner of death "*beyond all reasonable doubt*". Therefore, in human medicine, a range of ancillary tests have been proposed to confirm specific causes of death such as the *diatom test* for the diagnosis of drowning or a *seminal fluid test* for the diagnosis of Animal Sexual Abuse (ASA). Although the application of some of these tests has been sporadically described in veterinary medicine, their use in veterinary forensics practice requires additional rigorous validation studies. Therefore, the purpose of this thesis work is to summarize the studies carried out throughout the PhD scholarship, which were based on development of new methodological approaches in veterinary forensic medicine and pathology. Attention was paid to these sub-fields of the veterinary forensic medicine:

1) Forensic photography 2) Forensic traumatology 3) Post mortem interval 4) Diagnosis of drowning and 5) Forensic microbiology.

As regard the forensic photography, we assessed the suitability of “*Google Glass device*” in veterinary forensic photographic documentation; furthermore, in forensic traumatology field, we reported the first case of cardiac rupture following non-penetrating chest trauma (NCT) in a cat. Moreover, we presented unusual cases of cardiac rupture with NCT in two dogs. As regard the post mortem interval, we investigated the correlation between time since death and post mortem muscle proteins degradation in dog. In addition, as regard the diagnosis of drowning, we evaluated: 1) the macroscopic and microscopic findings in drowned animals and the contribution of necropsy and histological examination to determine the cause of death in drowning cases in veterinary forensic pathology 2) the differences in the number and location of diatoms between animals who died in drowning and nondrowning conditions and 3) the correlation between the time of permanence in water and the number and location of diatoms in animals dead for causes other than drowning and subsequently used for experimental drowning in standard conditions. Finally, in forensic microbiology field, we assessed the contribution of post-mortem microbiology in establishing a cause of death in young dogs who died of sudden and unexpected death. The results of my thesis showed that Google Glasses were usable in the veterinary forensic pathology of pet animals allowing a reduction in the mean execution time of necropsy and the acquisition of images useful for forensic documentation purposes. Furthermore, as regard the post-mortem modification of the muscles, we

observed a time depend post-mortem degradation of the muscle proteins such as desmin and dystrophin. In addition, as regard the diagnosis of drowning, we reported a statistically higher diatom number in the tissues of drowned animals than in the tissue of nondrowned animals and experimentally drowned cadavers. In contrast, similar macroscopic and histological injuries were observed in both nondrowned and drowned animals. Finally, as regard the forensic microbiology, we observed a high frequency of viruses and bacteria detected in cases of animals who died of sudden and unexpected death such as the following: *Canine Parvovirus type 2*, *Clostridium Perfringens* and *Canine Distemper Virus*. Together, these findings will provide useful tools to increase the knowledge in veterinary forensic medicine by reducing the acquisition of information from the human medical literature which, although very complete, does not provide information that are perfectly applicable to the species of veterinary interest.

I. Forensic veterinary medicine and pathology

The word “forensic” is defined in the Concise Oxford English Dictionary as meaning “*relating to, used in, or connected with a court of law*”. Thus, “*forensic veterinary medicine*” can be defined the “*branch of veterinary medicine that studies the applications of veterinary medical science to legal settings*”. The veterinary forensic pathology can be considered a subfield of the forensic medicine that deals with death investigations (Brooks, 2018). It is based on a transverse and multiorgan approach that includes *necropsy, histological examination, immunohistochemistry (IHC) and collateral examinations* such as *laboratory analysis and diagnostic imaging* to resolve obscure fatalities (Piegari et al. 2018). The duties of a veterinary forensic pathologist are similar to those of a human forensic pathologist and include: *crime scene investigation, evaluation of the clinic history of the animals, forensic necropsy, forensic histopathology, forensic photographic documentation and writing of the necropsy report*. The ultimate goal of this branch of veterinary medicine is to determine *the cause of the death of the animal*, whether natural or violent, *the manner of death, the time since death (post-mortem interval)* and *to examine and preserve any physical evidence* that might produce useful information for identifying and charging those guilty of crimes against animals (Brooks, 2018).

II. Animal law

Forensic Veterinary Medicine has its reason to exist, thanks to the laws that protect animals and make those who commit crimes against them liable to penalties or sanctions. In Italy, the most significant animal protection laws include:

- **Legislative Decree 157/92** Rules for the protection of homeothermal wild animals and for hunting purposes.
- **Legislative Decree 281/91** law on pets and the prevention of straying.
- **Legislative Decree 150/92** Regulation of offences relating to the application in Italy of the Convention on the international trade of endangered animal and plant species.
- **Legislative Decree 151/2007** Penalty provisions for violating the provisions of the Regulations (EC) no. 1/20055.
- **Legislative Decree 189/2004** Provisions concerning the prohibition of animal abuse, as well as the use of animals in clandestine fighting or unauthorised competitions.
- **Regulation (ec) 1523/2007** of the European Parliament and Council of 11 December 2007 banning the placing on the market and the import to, or export from, the Community of cat and dog fur, as well as products containing such fur.
- **Ministerial Ordinance of 18 December 2008** and its subsequent amendments: Guidelines on the prohibition of the use and keeping of poisoned baits.

- **Law 727 of the Italian Criminal Code** punishes the abandonment of animals.

Among these, **Law 189 introduced in 2004**, is one of the most important in the field of the veterinary forensic medicine. This law changed the legal basis for the protection of animals, which until then had been governed only by *Article 727* of the Criminal Code. With the amendment of this law, animal sentiment is now being harmed and no longer just the "human morality".

With Art. 1 "Amendments to the Criminal Code" of the aforementioned Law, the legislator introduced (after title IX) the title IX bis, entitled "**Crimes against animal sentiment**" into the Criminal Code. Following this change, animal abuse by just one offence becomes a crime.

This change involves:

- 1) an aggravation of penalties (from fines to imprisonment);
- 2) the impossibility of overturning the offence by means of an oblation;
- 3) the lengthening of the limitation period;
- 4) the necessity of willful misconduct, also in the form of the so-called "potential" misconduct (negligent misconduct is excluded from the regulations, except for the offence referred to in Article 727 of the Criminal Code).

The articles introduced by this law are:

-
- **544 bis**: killing of animals;
- **544 ter**: animal abuse;
- **544 quater**: prohibited shows or events using animals;
- **544 quinquies**: prohibition of animal fighting.

In particular, the articles of greatest interest for the forensic veterinary pathologist are **544 bis and 544 ter**. Art. 544 ter states the following:

***Par. 1** “Anyone who, for cruelty or without necessity, causes injury to an animal or subjects it to torture, hard labour or behaviour, or to unbearable work due to its ethological characteristics, shall be punished with imprisonment from 3 months to 1 year, or given a fine ranging from €3,000 to €15,000”*

***Par. 2** “The same punishment is applicable to anyone who administers prohibited or narcotic substances to animals or subjects them to treatments that cause damage to their health”*

***Par. 3** “The penalty is increased by half if the facts referred to in the first paragraph results in the death of the animal”*

From this article, it can be deduced that the action perpetrated constitutes a crime when the conscience and will to cause damage to the animal is present.

This damage, according to what has been mentioned above, may be of different types: *physical injury, torture, hard labour or behaviour, or unbearable work due to its ethological characteristics, administration of drugs or prohibited treatments that cause damage to the health of the animal*. The third paragraph of Article 544 provides for an aggravating circumstance, which materializes if the conduct referred to the first paragraph results in the death of the animal. Such an aggravating circumstance only exists if the death of the animal is an unintended consequence of the abuse. If this is not the case, the crime of killing animals is then committed as provided for in Article 544 bis of the Italian Criminal Code, pursuant to which:

"Whoever, whether cruelly or without necessity, causes the death of an animal shall be punished by imprisonment from three to eighteen months".

This law does not provide for:

- a distinction between owned or stray animal;
- the specific methods used to cause the death of the animal; both action and negligence, resulting in death, are punishable.

Finally, article 727c.c., reports the following:

Par. 1 *"Whoever abandons pets or animals that have acquired captive habits is liable to imprisonment for up to one year or a fine between €1,000 and €10,000."*

Par. 2 *“The same punishment is applicable to anyone keeps animals in conditions incompatible with their nature and producing severe suffering.”*

Here, we have two types of punishment: *the abandonment of animals and their keeping in conditions that conflict with their nature and cause suffering*. The concept of abandonment can be traced back to carelessness or neglect of the animal and not to cruelty to the animal or the infliction of gratuitous suffering, attitudes that are punished with the crime of animal abuse (*art. 544 ter*).

Abandonment, in any case, is not just a matter of abandoning the animal, but must be understood in the more general intention of no longer taking care of it. Furthermore, as regard the keeping of animals in conditions incompatible with their nature, it is not to be understood as a necessarily intentional offence, as it can be committed through negligence alone. Therefore, the keeper of animals in conditions incompatible with their nature or in a state of abandonment is criminally liable even through negligence alone. With regard to *"serious suffering"*, the Court of Cassation has specified that, *“while it is undeniably true that the concept of the gravity of the suffering necessary to fulfill the conduct described in art. 727 of the Italian Criminal Code is indeed different from the concept of serious damage to the animal's health provided for in art. 544 ter of the Italian Criminal Code, it is nevertheless essential that the suffering to which poorly kept animals should be subjected reach a level such as to make the condition in which they are kept absolutely irreconcilable with the proper conditions for the animal to be in a situation of well-being”*. This opinion should be expressed with reference to contingent situations, it being clear that a

temporary situation of distress for the animal cannot be confused with the “contra legem” situation set forth in paragraph 2 of art. 727.

III. Crime scene analysis

The crime scene can be divided in *primary crime scene*, *secondary crime scene and disposal site*. The primary crime scene is the *place where the most of the crime act was committed* or, more in detail, “*the place where offender engaged in the majority of his or her attack or assault upon the victim or victims*” (Turvey, 1999; Savino et al. 2011). In contrast, the secondary crime scene is “*any place where there are evidence of interaction between criminal and victim outside the primary scene*” (Turvey, 1999; Savino et al. 2005). Finally, disposal site is “*the place where the body of the victim is found*” (Savino et al. 2011). As a whole, a crime scene can be compared to a canvas of the expressionist period because of the set of disjointed information that a forensic pathologist must be able to acquire and subsequently relate to each other in order to reconstruct the initial situation which, exactly as in a canvas, produced the effects that then need to be analyzed. The complexity of a crime scene is inextricably linked to the unpredictability of human actions, which do not always follow a definite logic. The information obtained from the crime scene analysis is important not only for correctly interpreting the injuries observed during the forensic post-mortem examination but also to establish specific relationship between a suspect and the crime scene or victim (Gardner, 2011; Touroo and Fitch; 2016). Indeed, according to the Locard's exchange principle “*Every contact leaves a trace*”. In particular, *when two objects come into contact, there is*

always a mutual exchange of matter between them. Therefore, matter from the crime scene may be carried away on the criminal and, at the same time, criminal may live matter at the crime scene (Reddy, 2011). For these reasons, the work of the forensic pathologists should always begin with the analysis of the crime scene. Unfortunately, in Italy, the veterinary forensic pathologist hardly ever visits the crime scene. Although the pathologist may request photographs of the crime scene and law enforcement reports, the information that can obtain by examining the crime scene directly is irreplaceable. Indeed, in most cases, the law enforcement does not have specialist skills. Therefore, it is possible that important findings from a medical-legal point of view are not detected and consequently not photographed and collected. In general, the intervention of a pathologist on the crime scene is all the more useful the more promptly it is conducted.

Indeed, the biological materials, such as traces of blood or semen, deteriorate rapidly. Furthermore, with the increase of the time elapsed since death, post-mortem modifications are more influenced by environmental variables, such as *temperature, weather and presence or absence of shade.*

In general, all evidence obtained from a crime scene can be divided in 3 categories: **testimonial, physical and situational.**

Testimonial evidence can be defined as “*a written or oral statement made by witness or suspect to the law enforcement*” (Touroo and Fitch, 2016).

The physical evidence is “*any object that could be used to determine whether a crime has been committed or not, that could provide or disprove a link between a crime and victim or a link between a crime and a suspect*” (Touroo and Fitch, 2016). Finally, the situational evidence is “*any potentially transitory element on the crime scene, such as weather*

conditions, sounds and temperatures” (Touroo and Fitch, 2016). Since the statement made by witness or suspect can be easily altered, testimonial evidence should always be supported by specific physical or situational evidence. Finally, the evidence obtained during the evaluation of the crime scene should then be matched with the findings obtained from the forensic anatomopathological examination, histological analysis and collateral examinations. According to Toouro and Fitch (2016), we report in textbox 1 the main steps of the crime scene analysis in veterinary forensic medicine.

Textbox 1. Main steps of the crime scene analysis

1. photographic documentation of the crime scene upon arrive
2. photographic documentation of the environments and living conditions of the animal
3. evaluation of the presence of animals other than victim, if present, photographic documentation of the animal condition, execution of a brief forensic clinical examinations, identification and preservation of the evidences and rapid transport to the hospital
4. photographic documentation of the victim using a photomacrographic scale (ABFO No. 2 Standard Reference Scale, i.e.)
5. external examination of the victim, photographic documentation of the evidence on the surface of the body, preservation of the evidences.
6. estimate of the time since death
7. support to law enforcement for the identification and preservation of medical and nonmedical evidences (anabolic steroids or dogfighting paraphernalia, i.e.)

IV. Forensic necropsy

Necropsy, literally meaning “observation of the corpse”, is carried out for clinical (*diagnostic or “clinical” necropsy*) as well as for medico-legal purposes (*forensic necropsy*).

Diagnostic necropsy is performed to establish the *nature of the disease that has caused the death of the animals* in cases in which the ante-mortem diagnosis has failed. Furthermore, clinical necropsy can be carry out to *epidemiological purposes, to check and verify the diagnoses formulated in life or to study specific disease even when the cause of death is known* (Mason et al, 2007, Tariq et. al 2008, Kuker et al 2018, Brooks, 2018, Nwoha and Onoja; 2016) .

In contrast, the aims and objectives of forensic necropsy are very broad and include not only to determine *the cause of death*, but also to establish the *identity of the deceased*, *evaluate the manner of death*, *estimate time since death (post-mortem interval)* and *examine and preserve any physical evidence observed during the necropsy* (Brooks, 2018, Brooks, 2016, Touroo and Fitch; 2016).

Although the forensic necropsy technique is similar to that performed during a diagnostic necropsy, there are important differences in the approach and pathologist’s training (Brooks, 2018). Indeed, since the information obtained from the forensic necropsy is used for legal purposes, this procedure should be performed using previously established standard guidelines to avoid possible disputes during the process. Currently, the National protocols and the published international guidelines as well as the textbook of veterinary forensic pathology advocate a schematic, rigorous

and multidisciplinary approach to the investigation of all cases of veterinary forensic interest (Brooks et al. 2016, Brooks, 2018). In particular, the necropsy should consist of 2 components, specifically an “outer” and an “inner” necropsy (Piegari et al. 2018). The *outer necropsy* is the external examination of the body, *the inner necropsy* is the internal examination of the body and starts with the skinning of the cadaver (Piegari et al. 2018). However, the “outer” necropsy should not begin before a careful examination of the modalities and the conditions in which the cadaver has been received (Brooks, 2018). In particular, in the event that the cadaver is delivered inside a package, a radiographic examination should be performed before removing it from the packaging (Brooks, 2018). This procedure is useful to detect visible radiographically evidence (such as metal) that might be contained within the packaging. After this procedure, packaging and any physical evidence should be collected and preserved for any further laboratory test. Once removed from the package, the body should be radiographed to assess the presence of bone fractures, metallic objects in the animal body or other radiographically visible evidence (Brooks, 2018; Brooks et al. 2016). After radiographic examination, the body should be photographed from multiple angles using a photomacrographic scale (Brooks, 2018; Piegari et al., 2018). During this step, any evidence present on the surface of the body should be documented, photographed and subsequently sampled before any further handling of the cadaver (Brooks, 2018). The pathologist should document *species*, *breed* and *sex* of the animals, estimate *the time since death* and, if possible, *estimate the age of the victim*. (Brooks, 2018; Piegari et al., 2018). Subsequently, after a general evaluation of the body, a systematic evaluation of body parts of the animals

should be performed. For this purpose, *the head, mouth, mucous membranes, thorax, abdomen, perianal region, outer genitalia, hair coat, tail, as well as the front and hind limbs* should be surveyed. After this, a full skilling of the animals, inspection of the muscles and sub cutis as well as the opening of all body cavities (skull, chest, abdomen, pelvis) should be performed. Finally, all organs should be examined and dissected (Piegari et al. 2018). Each of these phases requires a careful and complete photographic documentation. Since necropsy is an unrepeatable procedure, the forensic photographic documentation should be accurate and detailed, but also produce a minimum delay in the execution time of the necropsy (Piegari et al. 2018). Forensic photography should be used to document both the presence of injuries (positive photograph) and the absence (negative photographs) of injuries (Dolinak et al. 2005). In addition, during images acquisition, a photomacrographic scale (*ABFO No. 2 Standard Reference Scale*, i.e.) placed near the injuries should be used to provide a geometrical reference in the forensic photographic documentation of the evidences (Brooks, 2018; Piegari et al., 2018). At the end of necropsy, representative tissue samples should be collected and fixed in 10% neutral buffer formalin for further histopathological examinations. In human forensic pathology, the National Association of Medical Examiners recommends to perform the histological examination on all forensic cases where the cause of death is not determined after the forensic macroscopic examination (Peterson and Clark; 2006). The histological examination of tissues can in fact provide a wide range of additional information that can help the pathologist to better interpret the injuries found during the necropsy. It may help to exclude other possible causes of death, confirm or deny the diagnosis formulated on the

macroscopic examination or allow the detection of specific injuries that cannot be observed on the forensic macroscopic examination (such as myocarditis and neurodegenerative diseases) (Brooks, 2018). According to Piegari et al. (2018), we report in textbox 2 the main steps of the forensic necropsy in veterinary forensic pathology.

textbox 2. Forensic necropsy protocol.

1. Victim identification procedures
2. Evaluation of thanatological aspects and estimation of the time elapsed since death
3. External examination of the body (state of nutrition, mucous membranes, body orifices, general conformation, superficial lesions, hair coat, external parasites, and teeth)
4. Skinning with evaluation of subcutis and muscles
5. Opening and evaluation of body cavities (skull, thorax, abdomen, and pelvis)
6. Extraction and general macroscopic evaluation of organs
7. Dissection of all organs
8. Specific evaluation of wounds or injuries
9. Complete photographic documentation of external appearance of the animals, body cavities, organs, and injuries

V. Postmortem interval in veterinary medicine

In common language and, more generally, for the usual clinical purposes, *clinical death* is defined as a permanent cessation of main vital cardiovascular, respiratory and neurological functions, which constitute the so-called "Bichat's tripod" (Kanstenbaum, 2006; Steffers and Credner; 2012). In contrast, *biological death* begins with the cessation of physiological processes that maintain the integrity and function of the cell (Steffers and Credner; 2012). In general, almost immediately after death, an irreversible and progressive sequence of physical and biochemical changes occurs in the body of cadavers. Normally, these changes have a well-defined order of progression; however, the rate of their onset and development is influenced by a wide range of variables that are both *environmental and intrinsic* to the subject itself, such as *weight, age, size, amount of adipose tissue, previous diseases and state of health of the animal before death, environment temperature, humidity and air flow*. A knowledge of the rate of development of postmortem changes is useful not only to better interpret the macroscopic and histological findings at necropsy but also to estimate the time since death, also called the "*postmortem interval (PMI)*" (Brooks; 2018). The postmortem interval is important in human forensic pathology and is equally important in veterinary forensic pathology. Determining the PMI can in fact provide law enforcement officials with useful information to include or exclude particular individuals in a group of suspects or possibly validate the testimony of a witness (Brooks; 2016). Unfortunately, although studies on the postmortem interval are one of the most popular topics of research in human forensic medicine, in veterinary medicine, published data

on postmortem changes and their rate of development are scarce (Brooks; 2018). A veterinary pathologist could try to estimate the PMI using data and parameters applied in human medicine. However, it is important to consider that most of these parameters have not been validated in the veterinary field. Therefore, they can not be use to estimate the post-mortem interval in animals of the veterinary forensic interest. Furthermore, it is important to bear in mind that the accuracy of the PMI estimate decreases appreciably with increasing time since death. Moreover, despite decades of research, no additional techniques have been introduced into human medicine to significantly increase the accuracy of a postmortem interval (Brooks, 2018; Perper; 2006; Swift; 2010). Thus, to estimate the PMI accurately, a wide range of post-mortem modifications should be evaluated, and the data obtained should be interpreted by analysing a wide range of variables both environmental and intrinsic to the subject itself. Here, we report the main postmortem changes evaluated in the forensic field for estimating the post mortem interval in both human and animals.

V.I Algor mortis

Algor mortis, literal meaning “*Coldness of death*”, is a normal and progressive cooling of the body that ends when the temperature of the cadavers equilibrates with environment temperature. This cooling is due to the cessation of the normal metabolic processes that maintain a baseline temperature during the life (Brooks, 2018). Currently, post-mortem temperature decay model is one of the most frequently used methods to estimate the PMI in human forensic pathology (Brooks, 2018). Indeed, many studies have been conducted in human medicine to obtain reference ranges useful for estimating the post-mortem interval. In contrast, only few studies have investigate the correlation between body temperature and time since death in veterinary forensic pathology (Kaliszar et al.; 2005; Kaliszar et al. 2009; Proctor et al. 2009; Erlandson, 2007). A veterinary pathologist could try to estimate the PMI using data and parameters applied in human medicine. However, the different size, weights and basal temperatures of humans compared to animals of veterinary interest make human temperature decay ranges of limited use in forensic veterinary field (Munro R and Munro HM, 2013; Munro R and Munro HM; 2008). Proctor et al. (2009), in a recent study conducted on 16 dead dogs stored for 32 h at temperature of 21.5 °C (range 20.7–22.7C, SD 0.55C) with relative humidity of 45% and mean air flow of 0.1 m, showed a reduction in rectal temperature of 0,5 degrees per hour and a reduction in liver temperature of 0.4 degrees per hour. In this study, the cooling was inversely proportional to the Body weight and body volume. In contrast, the rate of post-mortem temperature reduction was not affected by sex, body mass and hair coat density. These data should be

applied to estimate the time since death in veterinary forensic medicine; however, considering the limited sample size used for the experiment, caution should be used in their practical application.

V.II Livor mortis

Livor mortis, literal meaning “*bluish color of death*”, is a post mortem gravity depend accumulation of the blood in the lower portions of the body (*gravity depend area*) causing a purplish red discoloration of the soft tissues, skin and internal organ (Brooks, 2016). This post mortem modification is not only important to estimate the PMI, but also to obtain valuable information about the position taken by the body after death and, in some cases, information about the cause of death. Indeed, although hypostasis normally presents a purplish red color, in some cases, it can show a deviation from the normal color related to the victim’s cause of death, such as blue in asphyxiation or cherry red in deaths caused by carbon monoxide inhalation (Sorg, 1996). As regard the estimation of the time since death, few studies have investigated the rate of the development of hypostasis in animals of veterinary forensic interest (Erlandsson and Munro, 2007). In contrast, in human forensic pathology, the onset and the evolution of the livor mortis is well established. In general, in human medicine, it commonly develops between 30 minutes and 2 hours after death (Sorg, 1996). In this first phase, repositioning the body, the lividor can disappear completely from the primary site and reappear in the new gravity depend area. In addition, during this step, any object that pressing against the skin with sufficient pressure to

compress small blood vessel can cleared the lividor until the displaced blood is again permitted to flow back into the gravity-dependent areas (Brooks, 2016). This stage is referred as “non-fixed livor mortis”. Subsequently, about 8-12 hours after death, repositioning the body, only a partial migration of the lividor can be observed. Thus, changing the position of the body, lividors can be detected both in the original gravity depend area and in the new gravity depend area. This phase is referred as "partial migration of the livor mortis". Finally, at 24-48 hours post-mortem, hemolysis and decomposition of the blood vessel walls causes leaking of blood into the surrounding tissues. At this stage, lividor no longer disappear in response to mechanical pressure or to the movement of the cadaver. This phase is referred as "fixed livor mortis”

V.III Rigor mortis

Rigor mortis, letteral meaning “*the stiffening of death*”, is a biochemical reaction that determines a stable complex of adenosine and myosin of the muscle fibers causing a stiffening of the body's voluntary and involuntary muscles (Brooks, 2018). Immediately after the death, muscle fibers continue to consume adenosine triphosphate (ATP). In contrast, its synthesis is stopped (Shkrum and Ramsay, 2007). When the amount of ATP decreases to 85% of its normal level, actin and myosin begin to bind together stably and form a gel (Shepherd , 2003; Saukko, 2016). The maximum intensity and extension of the rigor mortis occurs when the ATP level decreases to 15% of its normal level (Saukko, 2016). Finally, the muscles stiffening

disappear with the onset of autolysis and putrefaction (Brooks, 2016; Brooks, 2018). The progression of rigor mortis is variable; however, in human medicine, precise intervals for the onset and resolution of post-mortem stiffness have been described in literature :

- start of rigor mortis at 1.5-4 hours post mortem
- complete rigidity at 12 hours after death
- maximum rigor intensity at 18-36 hours after death
- resolution at 24-50 hours after death

In human forensic medicine, the onset and progression of the rigor mortis is usually described according to a sequence of events known as the “*Nysten's rule*” (Di Luca and Feola, 2017). According to this rule, rigor mortis has a cranial-caudal progression, beginning with the palpebral and mandibular muscles, with subsequent extension first to the muscles of the upper limbs and then to those of the lower limbs. This first step is followed by stabilization phase during which all muscle fibers of the body remaining in a state of permanent stiffening. The last phase, that of resolution, always starts at the palpebral muscles and then extends in a cranio-caudal sense, first to the muscles of the upper limbs and finally to those of the lower limbs (Di Luca and Feola, 2017).

Unfortunately, in veterinary medicine, rates of development of rigor mortis and its resolution are not well-characterized. A study conducted on 11 beagle dogs stored at controlled temperature (range 11-18 °C) for 7 days showed a persistence of rigor mortis up to 7 days post-mortem (Erlandsson and Munro, 2007). However, the limited sample size used in the study,

associated with a lack of statistical analysis to analyze the data, make these findings not usable in forensic practice or during courts proceeding.

V.IV Dehydration

Post-mortem drying begins due to cessation of cardio-circulatory activity and the resultant lack of blood supply to the tissues. This loss of blood supply determines a progressive dehydration of mucous membranes and thinner skin surfaces first, and then of the internal organs. The speed of post-mortem drying of the tissues depends on intrinsic and extrinsic factors such as *environmental temperature, humidity and air flow*. Furthermore, post mortem drying is also responsible for some ocular modifications which, if opportunely evaluated, can provide useful information about the time elapsed since death. For example, ocular dehydration causes the formation of a horizontal red to brownish-black band on the sclera, called "*tache noir*". However, this modification is much more evident in humans than in animals because of the larger exposed sclera of human eye compared to that of animals (Brooks, 2018).

Unfortunately, in veterinary medicine, no study has investigated the rate of dehydration in animals and the ocular changes that result from it.

V.V Decomposition

Vertebrate corpse decomposition is the result of two mechanisms, **autolysis and putrefaction** (Dent et al., 2004; Janaway et al., 2009). Autolysis is defined as a “*cellular self-destruction process caused by hydrolytic enzymes contained within the cells*” (Shirley et al. 2011). In contrast, putrefaction can be defined as “*anaerobic decomposition of organic matter by the action of micro-organisms (bacteria, fungi and protozoa) resulting in the catabolism of tissue into gases, liquids and simple molecules*” (Vass, 2001; Dent et al., 2004; Brooks, 2016; Brooks, 2018). Over the years, many scales have been proposed to categorize the phases of decomposition in humans and animals, such as **Reed’s four-stage scale**, **Vass’s four-stage scale** (based on degree of decomposition), **Galloway’s five-stage scale** or **Wilson’s six-stage scale** (Brooks, 2016). Among these, **Galloway’s five general stages scale** can be used to better categorize the postmortem process of decomposition in nonburied vertebrate animals or in animals with a longer PMI. In contrast, Wilson’s six-stage scale can be used to better categorize the postmortem process of decomposition in buried animals or in animals with a shorter PMI (Brooks, 2016, Brooks, 2018). According to Galloway et al. (1989), the five-stage scale of decomposition can be summarized as follows:

- **Fresh:** *Visible changes caused by decomposition are limited: no discoloration of the tissues, no insect activity; (0-5 days p.m.)*
- **Early decomposition:** *grey to green discoloration of the soft tissues, insect activity, distention of the abdomen, hair loss, rupture of the*

skin and internal organs due to the buildup of pressure combined with the loss of tissues integrity (1-21 days p.m.)

- **Advanced decomposition:** *Liquefaction and disintegration of tissues, extensive insect activity, exposure of less than half of the bone surfaces (3 days- 18 months)*
- **Skeletonization:** *loss of readily available cadaveric materia; exposure of more than half of the bone surfaces; dry bone (13 days - 3 years)*
- **Extreme decomposition:** *skeletonization of the cadaver (2 months – 3 years)*

Unfortunately, the rate of decomposition is influenced by a broad range of variables. For example, **environmental characteristics** (*temperature, weather, presence or absence of shade*), **cadavers' intrinsic variables** (*open wounds, septicaemia, intracavitary effusion*) and the **action of micro and macrofauna** can dramatically affect the decomposition process. Overall, a large number of studies using pig cadavers have investigated the correlation between specific environmental or microenvironmental characteristics and cadaver decomposition rate. Archer (2004), in a study conducted on 20 newborn pig cadavers exposed to a damp forest setting, showed differences in the speed of body decomposition among seasons. In particular, this study showed a greater rate of decomposition in seasons with higher temperatures or in periods of greater rainfall. However, season and

rainfall are not the only environmental parameters that can influence the rate of decomposition. Indeed, within the same season, differences in the decomposition rate can also be observed between buried and nonburied animals. Willson et al. (2006), in a study conducted using pig cadavers buried in three upland habitats (*pasture, moorland, and deciduous woodland*), showed a relationship between the specific chemical conditions of the burial site and the body decomposition rate. Interestingly, the same study demonstrated that the process of decomposition itself can modify the burial microenvironment in terms of microbiological load, pH, humidity and changes in redox status. Overall, these findings suggested a direct influence of both burial site and cadaver on the body decomposition rate. Furthermore, a number of studies have focused on the influence of sun/shade on the rate of decomposition of a cadaver. In particular, in an experimental study conducted in Canada, a sample of 18 pigs were exposed either in the sun or in the shade in 3 different seasons (Sharanowski et al., 2008). This study showed differences in the rate of decomposition between sun/shade animals only in the spring, with faster decomposition of animals exposed to the sun than those exposed to the shade. Finally, a broad range of nonenvironmental variables can influence the rate of decomposition, such as **cause of death, insect activity, scavenger activity or trauma**. A study conducted using pigs showed a reduction in the rate of decomposition in clothed experimental carcasses compared to unclothed control cadavers (Card et al., 2015). This finding suggests that insect activity is an important variable that significantly influences the rate of decomposition. In addition, the process of putrefaction could be accelerated if there are certain antemortem conditions on the deceased. Sepsis, for example, can increase the bacterial

load in the corpse even prior to invasion of environmental microorganisms (Adams 2009; Zhou and Byard, 2011). In contrast, the relative sterility of newborns allows a slower than normal putrefactive process (Schotsmans et al., 2011). Finally, antemortem trauma was correlated with changes in the rate of decomposition in human forensic pathology studies. However, a study conducted on pigs did not show differences in the rate of decomposition between animals with external body trauma and control animals (Brooks, 2018).

V.VI *Histological changes*

Few studies have evaluated the correlations between post-mortem histological changes and time elapsed since death in animals of veterinary forensic interest. Overall, this method is considered of little use to estimate the post mortem interval because strongly influenced by a broad range of variables both environmental and subject's intrinsic. Under these premises, we report in table 1 the post-mortem histological modification assessed on 11 adult beagle dogs maintained at controlled temperature (range 11-18 °C) for 23 days (Erlandsson and Munro, 2007) .

Table 1. *Post-mortem histological changes*

	2 days	3 days	7 days	23 days
Lung	<i>limited loss of epithelium</i>	//	<i>complete loss of epithelium</i>	//
Heart	//	//	//	<i>Loss of myocardial nuclei</i>
Liver	//	//	<i>autolysis of most of hepatic nulei</i>	<i>Marked autolysis of hepatocytes</i>
Thyroid	<i>Mild to moderate autolysis</i>	<i>Mild to moderate autolysis</i>	<i>disruption of the normal architecture</i>	//
Tonsil	//	<i>Limited loss of the outer layers of the epithelium</i>	<i>Marked loss of the outer layers of the epithelium</i>	<i>Marked autolysis of the epithelium and lymphoid tissue</i>

V.VII Molecular methods

After an organism's death, RNA and DNA are degraded by ribonucleases and endonucleases present in the cell as well as by bacteria or other environmental sources. In general, DNA is more stable than RNA. Therefore, its degradation is slower compared to that of RNA. For this reason, the combined evaluation of RNA and DNA could theoretically provide useful information to better estimate the time since death.

Over the last years, many studies have investigated the correlation between time since death and post-mortem molecular degradation in laboratory animals, such as rat and mice (Zhu Y. et al. 2011; Fernanda et al. 2013; Zheng J et al. 2012). In contrast, to the best of our knowledges, only one study has investigated the RNA decay rate in dog. (Abdull-Azzez and Mustapha, 2017). This study, although very interesting and pioneering for the veterinary forensic pathology field, does not provide data applicable to the forensic practice due to the limited sample size (four in total) used in the study. Therefore, although this technique could bring future benefits for estimating the post-mortem interval, currently, this method can be considered of little use in veterinary forensic pathology.

V.VIII Radiographic modification

Few studies have evaluated the correlations between post-mortem radiographic changes and time elapsed since death in animals of veterinary forensic interest (Heng et al., 2009). However, this method is considered of little use to estimate the post mortem interval because strongly influenced by a broad range of variables both environmental and subject's intrinsic (Brooks, 2016).

VI. Main limitations in Veterinary Forensic Pathology

A broad range of injuries can be observed during forensic necropsy, such as the following:

- 1) *Injuries due to blunt force trauma;*
- 2) *Injuries due to penetrating trauma;*
- 3) *Thermal injuries;*
- 4) *Genital injuries due to animal sexual abuse (ASA);*
- 5) *Injuries due to drowning or nondrowning asphyxia;*
- 6) *Injuries due to electricity;*
- 7) *Injuries due to starvation.*

However, most of these injuries as well as the rate of the postmortem changes useful to estimate the time since death of animals have not yet been described in the veterinary literature. Therefore, the information for veterinary forensic medicine has often been acquired from the human forensic literature. This lack of information is currently considered a serious problem in veterinary forensic pathology. Indeed, although it is undeniably true that the mechanisms of lesions or postmortem modifications are similar between humans and animals, the different morphology, weight and tissue resistance of animals compared to human anatomy makes information validated in human forensic pathology not always applicable in the veterinary forensic medicine field. In addition, in human forensic medicine, macroscopic examination associated with histological analysis is often not sufficient to determine a victim's cause and manner of death "*beyond all*

reasonable doubt". Therefore, a range of ancillary tests have been proposed to confirm specific causes of death, such as the *diatom test* for the diagnosis of drowning or a *seminal fluid test* for the diagnosis of ASA (Brooks, 2018; Brooks, 2016). Although the application of some of these tests has been sporadically described in veterinary medicine, their use in veterinary forensics practice requires additional rigorous validation studies.

Therefore, the purpose of this thesis work is to summarize the studies carried out throughout the PhD scholarship, which were based on development of new methodological approaches in veterinary forensic pathology. Particular attention was paid to these sub-fields of the veterinary forensic medicine:

1. **Forensic Photography**
2. **Forensic Asphyxiology**
3. **Post Mortem Interval**
4. **Forensic Traumatology**
5. **Forensic Microbiology**

In particular, as regard forensic photography, we assessed the suitability of “*augmented reality*” in veterinary forensic photographic documentation; furthermore, in forensic traumatology, we reported and described 3 cases of cardiac rupture following non-penetrating chest trauma in cat and dog. As regard the post mortem interval, we investigated the correlation between time since death and post mortem muscle proteins degradation. In addition, as regard the forensic asphyxiology, we evaluated 1) the macroscopic and microscopic findings in drowned animals and the contribution of necropsy

and histological examination to determine the cause of death in drowning cases in veterinary forensic pathology 2) the differences in the number and location of diatoms between animals who died in drowning and nondrowning conditions and 3) the correlation between the time of permanence in water and the number and location of diatoms in animals dead for causes other than drowning and subsequently used for experimental drowning in standard conditions. Finally, as regard the forensic microbiology, we assessed the contribution of post-mortem microbiology in establishing a cause of death in young dogs who died of sudden and unexpected death.

- Abdul-Azeez I, Mustapha M, 2017. Post-Mortem Interval Estimation in Dogs: Hepatic Ribonucleic Acids Purity and Concentration Profile. *IMJ*, 48-50.
- Adams VI, 2009. Legal aspects of autopsy practice. In *Handbook of Autopsy Practice*, edited by Waters, B.L. Totowa: Humana Press Inc. pp. 137-143.
- Archer MS, 2004. Rainfall and temperature effects on the decomposition rate of exposed neonatal remains. *Sci Justice*, 44: 35–42.
- Brooks JW, 2016. Postmortem Changes in Animal Carcasses and Estimation of the Postmortem Interval. *Vet Pathol*, Sep;53(5):929-40.
- Brooks JW (ed.), 2018. *Veterinary Forensic Pathology*, 1st edition MD:Springer Verlag.
- Brownlie HW, Munro R, 2016. The Veterinary Forensic Necropsy: A Review of Procedures and Protocols. *Vet Pathol*, Sep;53(5):919-28.
- Card A, Cross P, Moffatt C, Simmons T, 2015. The effect of clothing on the rate of decomposition and Diptera colonization on *Sus Scrofa* carcasses. *J Forensic Sci*, 60(4):979–82.
- Dent B, Forbes S, Stuart B, 2004. Review of human decomposition processes in soil. *Environmental Geology*, 45(4): 576-585.
- Di Luca MN and Feola T, 2017. *Manuale di Medicina Legale*. 1th edition. Torino, Md: Minerva Medica.
- Dolinak D, Matshes E, Lew E, 2005. *Forensic Pathology: Principles and Practice*. 1th edition. San Diego, Md: Elsevier.

- Erlandsson M, Munro R, 2007. Estimation of the post-mortem interval in beagle dogs. *Sci Justice*, 47(4):150–154.
- Fernanda SS, 1 , Magalhães T, Carvalho F, Dinis-Oliveira RJ, Silvestre R, 2013. Profiling of RNA Degradation for Estimation of Post Mortem Interval. *PLoS One*, 8(2): e56507.
- Galloway A, Birkby WH, Jones AM, Henry TE, Parks BO, 1989. Decay rates of human remains in an arid environment. *J Forensic Sc*, 34(3):607–16.
- Gardner RM, 2011 *Practical Crime Scene Processing and Investigation*. 1st edition, Boca Raton, Florida MD: CRC Press.
- Gary LM, Dennis JM, 2007. Performing the Field Necropsy Examination. *Vet Clin North Am Food Anim Pract*, Nov;23(3):503-26
- Heng HG, Selvarajah GT, Lim HT, Ong JS, Lim J, Ooi JT, 2009. Serial postmortem thoracic radiographic findings in canine cadavers. *Forensic Sci Int*, Jul 1;188(1-3):119-24.
- Janaway RC, Wilson AS, Díaz GC, Guillen S, 2009. Taphonomic changes to the buried body in arid environments: An experimental case study in Peru. In *Criminal and Environmental Soil Forensics*, edited by Ritz, K., Dawson, L. & Miller, D. Bradford: Springer Science & Business Media. pp. 341-356
- Munro R, Munro HM, 2008. *Animal Abuse and Unlawful Killing: Forensic Veterinary Pathology*. Philadelphia MD:Saunders/Elsevier.
- Munro R, Munro HM, 2013. Some challenges in forensic veterinary pathology: a review. *J Comp Pathol*, 149(1):57–73.

- Nwoha RIO, Onoja IR, 2016. Necropsy Findings and Histopathological Changes in Dogs with Conjunct Experimental *Trypanosoma congolense*. *Br Microbiol Res*, 16(2): 1-8,
- Perper J, 2006. Time of death and changes after death. In: Spitz WU, Spitz DJ, editors. *Medicolegal investigation of death: guidelines for the application of pathology to crime investigation*. 4th ed. Springfield: Charles C. Thomas; p. 87–183.
- Peterson GF, Clark SC, 2006. Forensic autopsy—performance standards. *Am J Forensic Med Pathol*, Sep;27(3):200-25.
- Piegari G, Iovane V, Carletti V, Fico R, Costagliola A, De Biase D, Prisco F, Paciello O, 2018. Assessment of Google Glass for Photographic Documentation in Veterinary Forensic Pathology: Usability Study. *JMIR Mhealth Uhealth*. 6(9):e180.
- Proctor KW, Kelch WJ, New JC, 2009. Estimating the Time of Death in Domestic Canines *J Forensic Sci*, Nov;54(6):1433-7.
- Reddy KS, 2011. *The essential of forensic medicine and toxicology*. 30th edition, India, MD: K Sugana Devi.
- Saukko P, Knight B, 2016. *KNIGHT's Forensic Pathology*. 4th edition. Boca Raton: Taylor & Francis Group, LLC; pp. 62–6.
- Savino JO, Turvey BE, Baeza JJ, 2011. *Rape Investigation Handbook*. 2nd edition, MD: Academic Press.
- Schotsmans EMJ, Denton J, Dekeirsschieter J, Ivaneanu T, Leentjes S, Janaway, RC, Wilson AS, 2011. Effects of hydrated lime and quicklime on the decay of buried human remains using pig cadavers as human body analogues. *FSI*, 207(1): 51-59.

- Schotsmans EMJ, Denton J, Dekeirsschieter J, Ivaneanu T, Leentjes S, Janaway, RC, Wilson AS, 2011. Effects of hydrated lime and quicklime on the decay of buried human remains using pig cadavers as human body analogues. *FSI*, 207(1): 51-59.
- Simmons T, Cross PA, Adlam RE, Moffatt C, 2010. The influence of insects on decomposition rate in buried and surface remains. *J Forensic Sci*, 55(4):889–92.
- Sorg MH, Haglund WD, 1996. *Forensic Taphonomy: The Postmortem Fate of Human Remains*. 1st edition, MD: CRC Press.
- Steffers G, Credner S, 2012. *General Pathology and Internal Medicine for Physical Therapists*, 1 ed, TPS.
- Swift B, 2010. Methods of time since death estimation within the early post-mortem interval. *J Homicide Major Incident Invest*, 6(1):97–112.
- Shkrum MJ, Ramsay DA, 2007. *Forensic Pathology of Trauma*. 1st edition, Totowa, New Jersey: Humana Press Inc. pp. 24–8.
- Sharanowski BJ, Walker EG, Anderson GS, 2008. Insect succession and decomposition patterns on shaded and sunlit carrion in Saskatchewan in three different seasons. *Forensic Sci Int*, Aug 6;179(2-3):219-40.
- Shepherd R. Simpson's, 2003. *Forensic Medicine*. 12th edition, Euston Road, London: Arnold, a Member of the Hodder Headline Group; 2003. pp. 38–9.
- Shirley NR, Wilson RJ, Jantz LM, 2011. Cadaver use at the University of Tennessee's Anthropological Research Facility. *Clin Anat*, Apr;24(3):372-80.

- Simmons T, Cross PA, Adlam RE, Moffatt C, 2010. The influence of insects on decomposition rate in buried and surface remains. *J Forensic Sci*, Jul;55(4):889-92.
- Tariq KA, Chishti MZ, Ahmad F, Shawl AS, 2008. Epidemiology of gastrointestinal nematodes of sheep managed under traditional husbandry system in Kashmir valley. *Vet Parasitol*, Nov 25;158(1-2):138-43.
- Touroo R, Fitch A, 2016. Identification, Collection, and Preservation of Veterinary Forensic Evidence: On Scene and During the Postmortem Examination. *Vet Path*, Sep;53(5):880-7.
- Turvey B, 1999. *Criminal Profiling: An Introduction to Behavioral Evidence Analysis*, London: Academic Press.
- Vass AA, 2001. Beyond the grave-understanding human decomposition. *Microbiology Today*, 28(1): 190-19.
- Kastenbaum, Robert, 2006. "Definitions of Death". *Encyclopedia of Death and Dying*. Retrieved 27 January 2007.
- Kaliszan M, Hauser R, Kaliszan R, Wiczling P, Buczynski J, Penkowski M, 2005. Verification of the exponential model of body temperature decrease after death in pigs. *Exp Physiol*, sep;90(5):727–738.
- Kaliszan M, Hauser R, Kernbach-Wighton G, 2009. Estimation of the time of death based on the assessment of post mortem processes with emphasis on body cooling. *Leg Med (Tokyo)*, May;11(3):111–117.
- Küker S, Faverjon C, Furrer L, Berezowski J, Posthaus H, Rinaldi F, Vial F, 2018. The value of necropsy reports for animal health surveillance. *BMC Vet Res*, Jun 18;14(1):191.

Wilson AS, Janaway RC, Holland AD, Dodson HI, Baran E, Pollard AM, Tobin DJ, 2006. Modelling the buried human body environment in upland climates using three contrasting field sites. *Forensic Sci Int*, Jun 14;169(1):6-18.

Zheng J, Li X, Shan D, Zhang H, Guan D, 2012. DNA degradation within mouse brain and dental pulp cells 72 hours postmortem. *Neural Regen Res*, Feb 5; 7(4): 290–294.

Zhou C, Byard RW, 2011. Factors and processes causing accelerated decomposition in human cadavers-An overview. *Journal of Forensic and Legal Medicine*, Jan;18(1):6-9.

Zhu Y, Dong YC, Liang WB, Zhang L, 2011. [Relationship between RNA degradation and postmortem interval in mice]. *Fa Yi Xue Za Zhi*, Jun;27(3):161-3, 177.

Forensic Photography

Chapter 1

Assessment of Google Glass For Photographic Documentation in Veterinary Forensic Pathology

BASED ON: G. Piegari, V. Iovane, V. Carletti, R. Fico, A. Costagliola, D. De Biase, F. Prisco, O. Paciello, Assessment of Google Glass for Photographic documentation in veterinary forensic pathology: Usability study, JMIR mHealth and uHealth (2018), doi:<http://dx.doi.org/10.2196/mhealth.9975>.

1.1. Background

Google Glass is a device that was released for the first time as Google Glass explorer edition in 2013. It is a head-mounted device designed in the shape of a pair of eyeglasses with a 5.0-megapixel integrated camera; wireless connection; and the ability to take pictures, record a video, and call people with simple voice commands or manually by touching the frame. A small prism placed on the right side of the device allows a display of information to the user (Wei et al., 2018; Dougherty et al. 2017). As a whole, the multitasking capabilities of the device provide users a comfortable and multifunctional virtual experience. Although these advantages have not fully met the needs of private consumers, its voice control, wireless transmission capabilities, integrated camera, and app customization have attracted the interest of commercial industries and professionals from various fields, including the health care (Wei et al., 2018; Dougherty et al. 2017). In human medicine, Google Glass has been tested in many non-surgical fields such as on-demand data visualization and real-time analysis (Zhang et al., 2016), clinical simulations (Chaballout et al. 2016), management of diabetes (Wall et al. 2014), and pediatric cardiopulmonary resuscitation (Siebert et al. 2017). Furthermore, in neuropsychiatry, the usability and acceptability of Google Glass has been tested in children with autism spectrum disorder (Sahin et al. 2018). Similarly, in surgical settings, the multitasking capabilities of the device have allowed Google Glass to be tested in many surgical subfields such as cardiac surgery (Schaer et al. 2015), neurosurgery (Nakhla et al. 2017), orthopedics (Armstrong et al, 2014), general surgery (Hashimoto et al. 2016), and plastic surgery (Sinkin et al., 2016). In these studies, Google Glass has been used as a tool to monitor

vital signs, as an education instrument, and for telemonitoring and audiovisual recording. In human forensic pathology, Google Glass has been tested as a hands-free image acquisition device to document autopsies and postmortem examinations (Albrecht et al. 2014). However, to the best of our knowledge, despite the several publications in human medicine, no empirical evidence for using Google Glass in veterinary medicine settings is currently known. The aim of this study was to determine the suitability of Google Glass in veterinary forensic pathology by assessing (1) the difference in mean duration between necropsies conducted with Google Glass and a digital single-lens reflex (DSLR) camera (Nikon D3200, lens AF-S DX Nikon 18-55 mm f/3.5-5.6G VR), (2) the battery consumption during the necropsies, (3) the usability aspects, and (4) the quality of the photographic documentation of the Google Glass compared with a DSLR camera.

1.1.2 Image Acquisition in Veterinary Forensic Pathology

Photography is an important component of documentation in forensic pathology (Brownlei et al. 2016; Henham et al. 1994; Peterson and Clark, 2006). A correct and complete photographic documentation is also expressly required by the guidelines for forensic veterinary autopsies issued by the Italian Group of Forensic Veterinary Pathology. During the forensic necropsies, photography is important to document both the presence (positive photograph) and absence (negative photograph) of injuries (Dolinak et al. 2005); the main aim is the acquisition of images useful for legal purposes. Since necropsy is an unrepeatable procedure, the forensic photographic documentation should not only be accurate and detailed but also produce a minimal delay in the execution time of the necropsy (henham et al. 1994). Many DSLR cameras and mobile phones with photographic capabilities can be used for this purpose. However, these devices need to be used by qualified personnel with knowledge of photography and basics of veterinary forensic pathology in order to take clear and understandable pictures and minimize distortion and misleading information (Albrecht et al. 2014; henham et al. 1994; Senn and Stimson, 2010). Usually, this assignment is delegated to veterinary forensic pathologists themselves because there are not professional figures suited to this purpose. Furthermore, during autopsies, pathologists are forced to a continuous replacement of gloves so as to use cameras for documentary purposes. These limitations result in an excessive workload for the pathologists, with consequent lengthening of the time required to perform the necropsy. In this context, it is easy to imagine the advantage of having a device that allows taking hands-free pictures.

1.2. Methods

1.2.1. Forensic Necropsy Protocol and Image Acquisition

A total of 44 necropsies of 2 different species (22 dogs, 22 cats; dogs: medium-sized, age range 6-9 years, mean age 7.31 [SD 1.04] years; cats: age range 6-9 years, mean age 7.00 [SD 0.92] years) were performed by 2 pathologists (FP and GP) with training in forensic medicine. All necropsies were performed in the necropsy room of the Department of Veterinary Medicine and Animal Productions at the University of Naples Federico II, Naples, Italy, following a standard necropsy protocol summarized in Textbox 1 Each pathologist conducted 11 necropsies of both species. For each photographic acquisition, the images were taken using two different devices: Google Glass and a Nikon D3200 DSLR camera. During the external inspection of the body, in accordance with the guidelines for forensic veterinary autopsies, pathologists were asked to take pictures of the external appearance of the animals from many different angles. Furthermore, during the necropsies, pictures of organs before and after extraction and any other detail useful for documentation purposes was acquired. All images were acquired under standard lighting conditions and without using the internal flash of the camera. In addition, a standard background (blue table of 90 × 70 cm) was used to acquire pictures of organs and small anatomical details. Finally, during image acquisition, a photomacrographic scale (American Board of Forensic Odontology No. 2 Standard Reference Scale) placed near the injuries was used to provide a

geometrical reference in the forensic photographic documentation of the evidences.

Textbox 1 Forensic autopsy protocol.

- Victim identification procedures
- Evaluation of thanatological aspects and estimation of the time elapsed since death
- External examination of the body (state of nutrition, mucous membranes, body orifices, general conformation, superficial lesions, hair coat, external parasites, and teeth)
- Skinning with evaluation of subcutis and muscles
- Opening and evaluation of body cavities (skull, thorax, abdomen, and pelvis)
- Extraction and general macroscopic evaluation of organs
- Dissection of all organs
- Specific evaluation of wounds or injuries
- Complete photographic documentation of external appearance of the animals, body cavities, organs, and injuries

1.2.2 Evaluation of the Time of Necropsy and Battery Performance

To evaluate the differences in time of necropsy between autopsies conducted with Google Glass and DSLR camera and the battery performance of the devices, an additional number of 16 necropsies of 2 different species (8 cats and 8 dogs; dogs: medium-large dogs, age range 8-10 years, mean age 8.75 [SD 0.88] years; cats: age range 7-9 years, mean age 8.0 [SD 1.19] years) were performed by the 2 pathologists (FP and GP). In these cases, each pathologist conducted 4 necropsies of each species, with half of them conducted using Google Glass and another half using the Nikon D3200 DSLR camera. For each postmortem examination, we measured the time required to perform the necropsy using the stopwatch functionality available on a smartphone iPhone 6s Plus. To standardize the measurement, for all 16 forensic necropsies, the timer started at the first photographic acquisition and ended when the pathologist declared that he had acquired all pictures useful for the documentation purpose. Furthermore, each forensic examination began with devices (DSLR camera and Google Glass) charged to 100%, and at the end of each necropsy, the remaining battery power was noted.

1.2.2. Usability Aspect

At the end of each necropsy performed with the Google Glass, pathologists were interviewed to acquire information about the user experience. The questions were designed to obtain information about the usability aspects,

general experiences, and the main positive and negative features of the device.

1.2.3. Google Glass

The device—a Google Glass explorer edition—available during our study, ran on Android 4.4.2. specifications of the available developer explorer unit that included Texas Instrument OMAP 4430 SoC 1.2 GHz Dual core (ARMv7) processor, 2 GB of RAM and 12 GB of usable storage space, a 640×360 display, 802.11b/g Wi-Fi, Bluetooth, and a 5-megapixel camera [3,14]. It also had a 3-axis gyroscope, a 3-axis accelerometer, a 3-axis magnetometer, ambient light sensor, proximity sensor, bone conduction audio transducer, and 2 omnidirectional microphones (Figure 1)

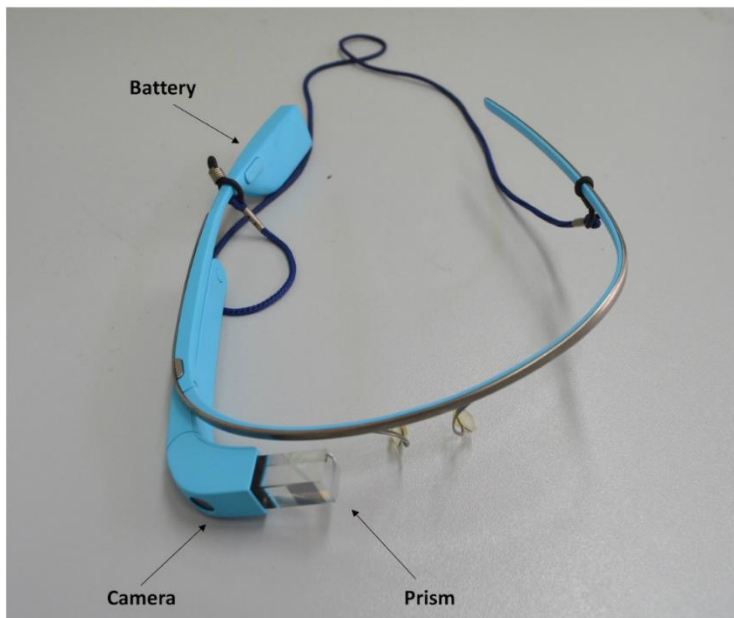


Fig. 1 Google glass device

1.2.4. Software Setup

For image acquisition, we used the preinstalled camera app for two reasons. First, we did not know whether the use of an app other than the preinstalled one would increase the battery consumption or decrease the photographic quality. Second, the voice commands and gestures performed on running the preinstalled app were easy to perform, intuitive, and precise; thus, we were not inclined to use an accessory app. However, to properly use the Google Glass camera during the necropsies, both pathologists followed a training course that lasted approximately 15 minutes. At the end of it, the pathologists declared to be able to use the device correctly. The voice commands used during our study were as follows: “show viewfinder,” to frame the anatomical region of interest correctly, and “take a picture,” to acquire the images. All accepted images were stored in a folder on the device until the pictures were transmitted via USB to an iOS-based laptop (MacBook Air 13”).

1.2.5. Digital Quality Image Assessment

A total of 5 forensic pathologists (AC, OP, RF, DD, and VI; 4 males, 1 female; age range 33-58 years, mean age 42.60 [SD 9.37] years) with a mean work experience of 19 (SD 7.41) years in the field of veterinary pathology, both diagnostic and teaching, were selected to evaluate the quality of the images taken with both devices. To avoid compromising the evaluation of the images by the memories of the necropsies, the pathologists selected for the evaluation of the images were different from those who physically

performed them. Furthermore, before the beginning of the evaluation, the pictures were divided into 3 groups: Group A, pictures of the external appearance of the animals; Group B, pictures of the organs; and Group C, pictures of small anatomical details or close-ups of the injuries. Each group included images taken with both devices. However, the device used for the acquisition was known (DSLR camera or Glass) only to the coordinators of the study. All 5 pathologists separately evaluated each group. In addition, all pictures were presented on a same computer (MacBook Air 13") with fixed display settings and similar environmental lighting conditions to avoid differences of evaluation due to external variables. The pathologists gave their opinions individually about the quality of the images analyzing 4 parameters: (1) overall color setting; (2) sharpness; (3) region of interest; and (4) brightness. Each one of these 4 parameters was separately evaluated on a 5-point score system according to Albrecht et al [14] (Table 1).

Table 1. Scoring system for image quality.

Parameter	Very poor	Poor	Average	Good	Very good
Sharpness	1	2	3	4	5
Overall color settings	1	2	3	4	5
Region of interest	1	2	3	4	5
Brightness	1	2	3	4	5

1.2.6. Statistical Analysis

Statistics were computed using SPSS Version 22.0 (IBM Corporation, 2014, Armonk, NY, United States). Student's t test was used to evaluate the difference in the mean duration between the necropsies conducted with Google Glass and the DSLR camera. The descriptive statistics for the ratings consisted of the tabulation of the frequency and percentages of scale items for each group for each item per device. To evaluate the differences between the ratings obtained for each group for both devices, we calculated an unpaired rank sum test (2-sided Mann-Whitney U test, with Cronbach alpha=.05). The same test was used to detect differences among the groups of the same device. To evaluate interrater reliability, we calculated intraclass correlation coefficients (ICCs) for the 5 pathologists for the items region of interest, sharpness, brightness, and color discrimination (Koch et al. 1982).

1.3. Results

1.3.1. Digital Image Quality Assessment

During the 44 forensic autopsies, the pathologists took 985 pictures with Google Glass and 985 pictures with the D3200 DSLR camera (504 photos of dogs and 481 photos of cats with each device). Each picture was evaluated by 5 pathologists, resulting in 4925 single evaluations each for Google Glass and the DSLR camera. Table 2 summarizes the results of the absolute frequencies and percentages of the ratings obtained per group for both devices for each of the 4 assessed parameters. The ratings of the images taken with Google Glass during necropsies were significantly lower than

those of the images acquired with the DSLR camera (Table 3). In particular, considering the percentage values, most ratings of the images taken with DSLR camera were high (good or very good) for all 4 parameters assessed. In contrast, for the images of Group A taken with Google Glass, the sum of frequency of ratings 5 (very good) and 4 (good) was 77.3% (1390/1800), 66.4% (1195/1800), 70.4% (1268/1800), and 71.7% (1290/1800) for region of interest, sharpness, brightness, and overall color settings, respectively. Furthermore, the images of Group B taken with Google Glass received a sum of frequency of ratings 5 and 4 of 54.7% (823/1505), 55.7% (838/1505), 65.8% (990/1505), and 54.0% (813/1505) for region of interest, sharpness, brightness, and overall color settings, respectively. The lowest ratings were observed in the pictures of Group C taken with Google Glass, with a sum of frequency of ratings 5 and 4 of 21.1% (342/1602) for region of interest, 26% (421/1602) for sharpness, 35.5% (575/1602) for overall color settings, and 61.4% (995/1602) for brightness. In this group, the differences between devices were particularly noticeable for region of interest, overall color settings, and sharpness. With regard to region of interest, the sum of frequency of ratings 2 and 3 amounted to 4.6% (74/1602) for images acquired using the DSLR camera versus 78.9% (1278/1602) for those acquired using Google Glass. Similarly, for overall color settings, this sum was 4% (65/1602) for images acquired using the DSLR camera versus 64.5% (1045/1602) for those acquired using Google Glass. Finally, for sharpness, the sum was 8.6% (140/1602) for images taken with DSLR versus 74% (1199/1602) for those taken with Google Glass. Furthermore, with regard to the pictures taken with Google Glass, statistical differences were observed in the distribution of ratings

between Group A and Group B and between Group C versus B and A for all 4 assessed parameters (Table 4)

1.3.2. Evaluation of Battery Performance and Time of Necropsy

Of the 16 necropsies conducted with Google Glass (8 necropsies) and DSLR camera (8 necropsies), we observed a reduction in the time of necropsy with Google Glass compared with that with the DSLR camera group. The mean duration of a single postmortem examination in the DSLR camera group was 126.38 (SD 3.46) minutes for the dogs group and 68.90 (SD 2.30) minutes for the cats group, whereas for the Google Glass, the mean duration was 111.11 (SD 3.29) minutes for dogs group and 55.5 (SD 2.06) for cats group. The differences were significant ($P < .01$). Furthermore, at the end of the necropsies conducted with Google Glass, the average percentage of battery power was 47% and 60% for the dogs and cats group, respectively (Table 5). For the DSLR camera, it was not possible to monitor the battery level because the display showed an icon with a crude scale in the unit of 33% and not the battery percentage. In any case, a significant battery consumption was not detected.

Table 2. Frequencies and percentages of evaluations given by 5 pathologists for images taken during forensic necropsies with Google Glass and Nikon D3200 reflex camera stratified for each group.

Parameters assessed and Score ^a	Group A, n (%)		Group B, n (%)		Group C, n (%)	
	Glass (n=1800)	DSLR ^b camera (n=1800)	Glass (n=1505)	DSLR camera (n=1505)	Glass (n=1602)	DSLR camera (n=1602)
Region of interest						
5	426 (23.7)	933 (51.8)	173 (11.5)	522 (34.7)	55 (3.4)	1027 (63.4)
4	964 (53.6)	803 (44.6)	650 (43.2)	898 (59.7)	287 (17.7)	519 (32.0)
3	393 (21.8)	64 (3.6)	622 (41.3)	85 (5.6)	827(51.1)	74 (4.6)
2	17 (0.9)	0 (0)	60 (4)	0 (0)	451 (27.8)	0 (0)
1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Total sharpness						
5	353 (19.6)	1005 (55.8)	37 (2.5)	434 (28.8)	125 (7.7)	762 (47)
4	842 (46.8)	708 (39.4)	801 (53.2)	929 (61.7)	296(18.3)	718 (44.4)
3	580 (32.2)	87 (4.8)	597 (39.7)	142 (9.4)	735 (45.4)	140 (8.6)
2	25 (1.4)	0 (0)	70 (4.6)	0 (0)	464 (28.6)	0 (0)
1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Overall color settings						
5	268 (14.9)	912 (50.7)	100 (6.6)	852 (56.6)	2 (0.1)	813 (50.2)
4	1022 (56.8)	783 (43.5)	713 (47.4)	619 (41.1)	573 (35.4)	742 (45.8)
3	507 (28.1)	105 (5.8)	582 (38.7)	34 (2.3)	770 (47.5)	65 (4.0)
2	3 (0.2)	0 (0)	110 (7.3)	0 (0)	275 (17)	0 (0)
1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Brightness						
5	285 (15.8)	997 (55.4)	204 (13.6)	680 (45.2)	125 (7.7)	880 (54.3)
4	983(54.6)	688 (38.2)	786(52.2)	806(53.6)	870 (53.7)	645 (39.8)
3	482 (26.8)	115 (6.4)	494 (32.8)	19 (1.3)	513 (31.7)	95 (5.9)
2	50 (2.8)	0 (0)	21 (1.4)	0 (0)	112(6.9)	0 (0)
1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

^aParameters were assessed on a scale of 1-5 (1, very poor; 2, poor; 3, average; 4, good, 5, very good).

^bDSLR: digital single-lens reflex.

Table 3. Unpaired rank sum, 2-sided Mann-Whitney U (Cronbach alpha=.05) for ratings of images taken with Google Glass and Nikon D3200 reflex camera for each group for each of the 4 assessed parameters

Assessed parameters	Group A		Group B		Group C	
	z score	P value	z score	P value	z score	P value
Region of interest	-20.211	<.001	-26.771	<.001	-44.398	<.001
Sharpness	-26.468	<.001	-29.412	<.001	-37.705	<.001
Overall color settings	-25.177	<.001	-38.475	<.001	-40.931	<.001
Brightness	-26.786	<.001	-28.283	<.001	-31.452	<.001

Table 4. Unpaired rank sum, 2-sided Mann-Whitney U (Cronbach alpha=.05) for ratings of the images of the 3 groups taken with Google Glass.

Assessed parameters	Group A versus B		Group B versus C		Group C versus A	
	z score	P value	z score	P value	z score	P value
Region of interest	-14.577	<.001	-22.603	<.001	-34.101	<.001
Sharpness	-11.396	<.001	-18.256	<.001	-26.095	<.001
Overall color settings	-12.740	<.001	-12.515	<.001	-25.412	<.001
Brightness	-2.737	.006	-5.052	<.001	-8.005	<.001

Table 5. Descriptive statistics of loss of battery consumption during forensic necropsy stratified by the device used to acquire the images: a Nikon D3200 reflex camera and the Google Glass device.

Device	Mean (SD)
Digital single-lens reflex camera	N/A ^a
Glass (dogs group)	47 (2.6)
Glass (cats group)	60 (2.9)

^aN/A: not applicable.

1.3.3. Usability

Based on interviews conducted at the end of the postmortem examination, we obtained subjective assessments about the user experience of Google Glass. As a positive aspect, the voice control was reported as useful, particularly in cases where both hands were occupied. The use of voice control led to increased saving of rubber gloves because the pathologists were not forced to take them off whenever they needed to take some pictures. In addition, the pathologists agreed about the ergonomics of the device and its lightness, which makes it comfortable to wear. As negative aspects, they reported the short battery life and difficulty to capture the desired regions of interest, especially for the close-ups. During use, the

device was placed on the head and there was no zoom function available. For these reasons, the pathologists were forced to place themselves too close to the dissection table to be able to take pictures of small anatomical details or close-ups of the injuries correctly (Figure 2).



Figure 2. The forensic pathologist wears Google Glass and takes pictures of small anatomical details in a hands-free manner.

1.3.4. Interrater Reliability

The interrater reliability was high. The ICC for the ratings obtained based on the forensic necropsy pictures indicated a good positive relationship for overall color settings (0.815, 95% CI 0.771-0.853; $P < .001$), region of interest (0.787, 95% CI 0.750-0.819; $P < .001$), sharpness (0.711, 95% CI 0.632-0.775, $P < .001$), and brightness (0.822, 95% CI 0.777-0.860; $P < .001$).

1.4. Discussion

1.4.1. Principal Findings

Potentially disruptive technologies such as Google Glass create excitement for the possible applications that they can have in health care; however, the use of new tools should be thoroughly evaluated and validated before applying them in the medical or biomedical fields. Google Glass has been tested in many medical fields such as clinical simulations (Chaballout et al. 2016), surgery (Schaer et al. 2015; Nakhla et al. 2017; Hashimoto et al., 2016), and neuropsychiatry (Sahin et al., 2018), but to the authors' knowledge, this is the first study to assess the potential of Google Glass in veterinary medicine. Both devices tested in our study achieved the set goals and both allowed a complete photographic documentation. However, differences in efficiency between the two devices were observed. Our study showed a reduction in the necropsy time in forensic examinations conducted with Google Glass compared with those conducted with the Nikon D3200 DSLR camera; this was because Google Glass allowed hands-free operation, avoiding the continuous replacement of gloves that is necessary during necropsies performed with a reflex camera. Although glove saving was not a parameter directly evaluated in this study, this aspect was highlighted by both pathologists during the interviews conducted at the end of the necropsies. In addition, we observed a rapid reduction in the battery life of the Google Glass. In our opinion, power consumption was not a limiting factor because at the end of the necropsies, the average battery percentage was 47% and 60% for the dogs and cats groups, respectively.

However, problems could arise in the case of more autopsies being performed on the same day. In these cases, pauses to allow battery charging should be considered. The difference in average Google Glass power consumption observed between the groups could be due to the different mean duration of a single postmortem examination of the dogs group (111.11 minutes) compared with the lower duration of the cats group (55.5 minutes) and, consequently, the mean duration of Google Glass use. Regarding the DSLR camera, it was not possible to monitor the battery level because the display showed an icon with a crude scale in units of 33% and not the battery percentage. In any case, significant loss of battery power was not detected. This is understandable considering the high capacity of the batteries commonly used in the modern DSLR cameras. Finally, images taken with Google Glass received significantly lower ratings for all 4 assessed parameters than those taken with the DSLR reflex camera. Most ratings for the images taken with the DSLR camera were high (good or very good) for all 4 assessed parameters. In contrast, in the pictures of the Group A taken with Google Glass, the sum of the frequency of ratings 5 (very good) and 4 (good) was 77.3% (1390/1800), 66.4% (1195/1800), 70.4% (1268/1800), and 71.7% (1290/1800) for region of interest, sharpness, brightness, and overall color settings, respectively; furthermore, the images of Group B taken with Google Glass received a sum of the frequency of ratings 5 and 4 of 54.7% (823/1505), 55.7% (838/1505), 65.8% (990/1505), and 54.0% (813/1505), respectively. The lowest ratings were observed for the pictures of group C with a sum of the frequency of ratings 5 and 4 of 21.1% (342/1602) for region of interest, 26.0% (421/1602) for sharpness, 35.5% (575/1602) for overall color settings, and 61.4%

(995/1602) for brightness. This finding could be explained considering the lower camera resolution of Google Glass (5-megapixel camera with a fixed focal length of 3 mm) compared with the DSLR camera (24 megapixel) (Aldaz et al., 2015). Hashimoto et al. (2016), in a recent study, reported that the video quality of iPhone 5 was greater than that of Google Glass during human surgical telementoring sessions. In contrast, some eye surgeons have reported a good video quality of the Google Glass device during scleral buckling surgeries (Rahimy et al., 2015). These findings suggest that the camera quality of Google Glass is evaluated differently depending on the medical field of application and, consequently, on the photographic and recording quality required. Specifically, in our study, the gradual reduction in good or very good ratings observed among Groups A, B, and C could reflect the progressive increase in photographic quality required for the evaluation of anatomical details compared with that required for the external evaluation of the body or organs. Albrecht et al. (2014), in a study conducted to assess the quality of the photographic documentation of the Google Glass device in human forensic pathology, showed a lower picture quality of the images taken with Google Glass than of those taken with a DSLR camera. In particular, the authors found that the differences between the devices were particularly noticeable only for region of interest and sharpness, whereas brightness and overall color settings showed similar distributions of ratings, with results slightly in favor of the pictures taken with the DSLR camera. These results appear in apparent contradiction with those obtained in our study. However, in this previous study, the images were evaluated as a whole and without being divided by type (external appearance, organs, and anatomical details). Furthermore, in the same study, an external application

was used for image acquisition and a lower number of pictures was evaluated. However, excluding the methodological differences, the different results obtained in this study could be explained considering the different size of the organs of pet animals compared with human anatomy. These differences suggest a greater difficulty in the evaluation of images of organs and injuries of pet animals than that of humans. Similarly, for the external examination of the body, the difference in the size of animals compared with human anatomy and the presence of hair, common in all species of veterinary interest but absent in humans, makes the qualitative camera differences between devices more evident in animals than in humans. In addition, in our study, regarding the images taken with Google Glass, statistical analysis showed differences between the groups for all 4 assessed parameters. However, the highest frequency of rating 2 (poor) was observed for the pictures of Group C. The lower ratings observed for region of interest in Group C compared with those in Groups A and B was the most important aspect, and it could be explained considering that the Google Glass device had a wide-angle lens but not a zoom function (Hashimoto et al., 2016; Aldaz et al., 2015). During the forensic necropsy, the field of view of the Google Glass camera was too large, which forced the pathologists to place themselves too close to the dissection table to acquire pictures of small anatomical details. This led to the acquisition of poorly framed images that were unsuitable for forensic documentary purposes. Similarly, Moshtaghi et al. (2015), in a study conducted to assess the feasibility of using Google Glass during otorhinolaryngological procedures, found that the image quality was inadequate for viewing small and deep-seated anatomical structures. However, in our opinion, although this aspect has already been

observed in human medicine, it could be even more relevant in pet animals than humans because of the differences in size between these species.

1.5. Limitations

A few limitations of the study should be noted. First, we were able to test the device with only 2 pathologists. However, this allowed a more accurate assessment of the necropsy execution times. In our opinion, a greater number of pathologists would have determined a high variability in the time of necropsy due to the different levels of manual dexterity of each pathologist. Second, for the evaluation of the images, this study was based on subjective opinions of raters and not on objective and reproducible parameters. However, to reduce this limitation, a high number of highly qualified pathologists was selected for the evaluation of the images. In addition, the ICCs were evaluated for the 5 raters for the items region of interest, sharpness, brightness, and color discrimination. Furthermore, this study was conducted only on cats and dogs. We decided to test the device on these animals because although diagnostic necropsies are commonly performed on a broad range of animals, at present, they are the main species of forensic interest in the veterinary field (Laricchiuta et al. 2018; Frank et al., 2015). However, this limitation makes these findings nonreproducible on other species of veterinary interest such as mice, rats, rabbits, or zoo and farm animals. Finally, the joint evaluation of the acquired images from 2 different species could be a further limitation of this study. However,

considering the similar morphology and size of these animals (medium-sized dogs vs adult cats), we do not believe this to be a limitation.

1.6. Conclusions

These findings suggest that Google Glass is usable in the veterinary forensic pathology of pet animals, but its image quality is lower than that of a reflex camera. In particular, the image quality of Groups A and B seemed adequate for forensic photographic documentation purposes. However, in this step of development, the high frequency of poor ratings observed for the pictures of Group C suggest that the device is not suitable for taking pictures of small anatomical details or close-ups of the injuries. In our opinion, the combined use of the two devices, reflex camera for capturing images of small anatomical details and Google Glass for capturing images of the external appearance of the animals and organs, could reduce the execution times of the necropsy, lead to considerable saving of gloves, and allow acquisition of pictures useful for forensic documentation purposes. However, further studies will be needed to evaluate the application of this device to other species of veterinary interest such as wildlife or farm animals. In some of these species, the greater volume of the organs than that in pet animals could make the qualitative differences between Google Glass and the reflex camera less evident but, above all, could make the absence of the photographic zoom in Google Glass less limiting.

Albrecht U, von JU, Kuebler J, Zoeller C, Lacher M, Muensterer OJ, et al., 2014. Google Glass for documentation of medical findings: evaluation in forensic medicine. *J Med Internet Res*,16(2):e53

Aldaz G, Shluzas LA, Pickham D, Eris O, Sadler J, Joshi S, et al., 2015 Hands-free image capture, data tagging and transfer using Google Glass: a pilot study for improved wound care management. *PLoS One*, 10(4):e0121179

Aquila I, Di NC, Paciello O, Britti D, Pepe F, De LE, et al., 2014. An unusual pedestrian road trauma: from forensic pathology to forensic veterinary medicine. *Forensic Sci Int* 2014 Jan;234:e1-e4

Armstrong DG, Rankin TM, Giovinco NA, Mills JL, Matsuoka Y, 2014. A heads-up display for diabetic limb salvage surgery: a view through the google looking glass. *J Diabetes Sci Technol*, Sep;8(5):951-956

Brownlie HWB, Munro R, 2016. The Veterinary Forensic Necropsy: A Review of Procedures and Protocols. *Vet Pathol* 2016 Dec;53(5):919-928.

Chaballout B, Molloy M, Vaughn J, Brisson IR, Shaw R, 2016. Feasibility of Augmented Reality in Clinical Simulations: Using Google Glass With Manikins. *JMIR Med Educ* 2016 Mar 07;2(1):e2

Dolinak D, Matshes E, Lew E, 2005. *Forensic Pathology: Principles and Practice*. San Diego: Elsevier Science

Dougherty B, Badawy SM, 2017. Using Google Glass in Nonsurgical Medical Settings: Systematic Review. *JMIR Mhealth Uhealth*, Oct 19;5(10):e159

Google. Glass: Frequently Asked Questions-Tech Specs URL: https://support.google.com/glass/answer/3064128?hl=en&ref_topic=30633
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Frank C, Madden DJ, Duncan C, 2015. Field Necropsy of the Horse. *Vet Clin North Am Equine Pract*, Aug;31(2):233-245

- Gerdin JA, McDonough SP, 2013. Forensic pathology of companion animal abuse and neglect. *Vet Pathol*, Nov;50(6):994-1006.
- Hashimoto DA, Phitayakorn R, Fernandez-del CC, Meireles O, 2016. A blinded assessment of video quality in wearable technology for telerenting in open surgery: the Google Glass experience. *Surg Endosc*, Jan;30(1):372-378
- Henham AP, Lee KA, 1994. Photography in forensic medicine. *J Audio Media Med*, Jan;17(1):15-20
- Koch G. Intraclass correlation coefficient. In: Kotz S, Johnson NL. editors. *Encyclopedia of Statistical Sciences*, 4th ed. New York: John Wiley & Sons; 1982:213-217.
- Laricchiuta P, Russo V, Costagliola A, Piegari G, Capasso M, Silvestre P, et al, 2018. Histological and immunohistochemical characterization of uterine adenocarcinoma in an Asian elephant (*Elephas Maximus*). *Folia Morphol (Warsz)*, Mar 23
- Mann H, Whitney D, 1947. On a test of whether one of two random variables is stochastically larger than the other. *Ann Math Statist*,18(1):50-60.
- McEwen BJ, Gerdin J, 2016. Veterinary Forensic Pathology: Drowning and Bodies Recovered From Water. *Vet Pathol*, Dec;53(5):1049-1056.
- McEwen BJ, 2012. Trends in domestic animal medico-legal pathology cases submitted to a veterinary diagnostic laboratory 1998-2010. *J Forensic Sci*, Sep;57(5):1231-1233.
- Millins C, Howie F, Everitt C, Shand M, Lamm C, 2014. Analysis of suspected wildlife crimes submitted for forensic examinations in Scotland. *Forensic Sci Med Pathol*, Sep;10(3):357-362
- Moshtaghi O, Kelley KS, Armstrong WB, Ghavami Y, Gu J, Djalilian HR, 2015. Using Google Glass to solve communication and surgical education challenges in the operating room. *Laryngoscope*, Oct;125(10):2295-2297.

Munro HM, Thrusfield MV, 2001. 'Battered pets': non-accidental physical injuries found in dogs and cats. *J Small Anim Pract*, Jun;42(6):279-290.

Nakhla J, Kobets A, De LGRR, Haranhalli N, Gelfand Y, Ammar A, et al., 2017. Use of Google Glass to Enhance Surgical Education of Neurosurgery Residents: "Proof-of-Concept" Study. *World Neurosurg*, Feb;98:711-714

Peterson GF, Clark SC, National Association of Medical Examiners. Forensic autopsy performance standards. *Am J Forensic Med Pathol* 2006 Sep;27(3):200-225.

Rahimy E, Garg SJ, 2015. Google Glass for Recording Scleral Buckling Surgery. *JAMA Ophthalmol*, Jun;133(6):710-711

Wall D, Ray W, Pathak RD, Lin SM, 2014. A google glass application to support shoppers with dietary management of diabetes. *J Diabetes Sci Technol*, Nov;8(6):1245-1246

Wei NJ, Dougherty B, Myers A, Badawy SM, 2018. Using Google Glass in Surgical Settings: Systematic Review. *JMIR Mhealth Uhealth*, Mar 06;6(3):e54

Wobeser G, 1996. Forensic (medico-legal) necropsy of wildlife. *J Wildl Dis*, Apr;32(2):240-249

Sahin NT, Keshav NU, Salisbury JP, Vahabzadeh A, 2018. Second Version of Google Glass as a Wearable Socio-Affective Aid: Positive School Desirability, High Usability, and Theoretical Framework in a Sample of Children with Autism. *JMIR Hum Factors*, Jan 04;5(1):e1

Salem NH, Belhadj M, Aissaoui A, Mesrati MA, Chadly A, 2013. Multidisciplinary approach to fatal dog attacks: a forensic case study. *J Forensic Leg Med*, Aug;20(6):763-766

Schaer R, Salamin F, Jimenez DTOA, Atzori M, Muller H, Widmer A, 2015. Live ECG readings using Google Glass in emergency situations. *Conf Proc IEEE Eng Med Biol Soc* 2015;2015:315-318

Senn D, Stimson P. *Forensic Dentistry*, Second Edition. Boca Raton: Taylor & Francis, Inc; 2010.

Sinkin JC, Rahman OF, Nahabedian MY, 2016. Google Glass in the Operating Room: The Plastic Surgeon's Perspective. *Plast Reconstr Surg*, Jul;138(1):298-302

Siebert JN, Ehrler F, Gervais A, Haddad K, Lacroix L, Schrurs P, et al., 2017. Adherence to AHA Guidelines When Adapted for Augmented Reality Glasses for Assisted Pediatric Cardiopulmonary Resuscitation: A Randomized Controlled Trial. *J Med Internet Res*, May 29;19(5):e183

Stern AW, Smith-Blackmore M, 2016. Veterinary Forensic Pathology of Animal Sexual Abuse. *Vet Pathol*, Dec;53(5):1057-1066

Zhang YS, Busignani F, Ribas J, Aleman J, Rodrigues TN, Shaegh SAM, et al, 2016. Google Glass-Directed Monitoring and Control of Microfluidic Biosensors and Actuators. *Sci Rep*, Mar 01;6:22237

Forensic Asphyxiology

Chapter 2

Diagnosis of Drowning in Veterinary Forensic Pathology

BASED ON: Giuseppe Piegari, Angelo Genovese, Rosario Fico, Francesco Prisco, Orlando Paciello, Diagnosis of Drowning in Veterinary Forensic Pathology, Sisvet meeting, 2018

2.1. Drowning: definition and mechanism of death

The World Health Organization (WHO) defines drowning as a “*process of experiencing respiratory impairment from submersion/immersion in liquid*” (Beeck et al, 2005). Furthermore, according to the WHO, drowning outcomes are currently classified as *death, morbidity and no morbidity* (Beeck et al., 2005). This new definition has replaced the previous classifications of drowning (Idris et al, 2003). Thus, the terms “*wet*”, “*dry*”, “*active*”, “*passive*”, “*silent*” and “*secondary*” are not currently recommended to describe drowning cases (McEwen and Gerdin, 2016). Overall, the main mechanism of drowning is rapid and persistent hypoxemia due to the introduction of water or other liquids into the airway (Conn et al, 1995; Layon and Modell, 2009; Lunetta and Modell, 2005, McEwen and Gerdin, 2016). However, to fully understand the breadth and chronology of the physiological and biochemical changes that occur during “*drowning*”, a familiarity with the “*drowning process*” is essential. Unfortunately, the veterinary literature on macroscopic and microscopic changes following accidental and nonaccidental drowning is scarce (McEwen and Gerdin, 2016, Banting et al. 1938). However, previous studies conducted using experimental animal models of drowning provide useful information about physiological and biochemical changes in response to drowning in veterinary medicine (Bhardwaj, 1982; Banting et al. 1938). Overall, the drowning process is a complex mechanism that involves a sequence of events such as *cardiorespiratory and vagal reflexes, electrolyte modifications* and *hypoxemia* with subsequent *neurological and cardiological dysfunction* that can eventually culminate in *death of the*

subject (McEwen and Gerdin, 2016). Usually, the first step of drowning is vagal-mediated laryngospasm due to contact between larynges and water or other liquids. Previous studies have shown that aspiration of only 2 mL of water can cause a laryngeal spasm in dogs (Banting et al. 1938). This condition lasts for approximately 2-3 minutes and is associated with an increase in systemic CO₂ and the development of metabolic acidosis and bradycardia (McEwen and Gerdin, 2016). During this first step, laryngospasm may prevent the entrance of water into the lungs. The persistence of laryngospasm during drowning was used in the earliest drowning studies to explain the macroscopic findings of “dry lungs” observed in approximately 10–20% of drowning cases in human forensic pathology (Lunette, 2005; Modell et al., 1999). Although a relationship between laryngospasm and “dry lungs” might seem logical, previous studies suggested that “dry lung” could be a consequence not only of the laryngospasm but also of vago-vagal cardiac inhibition, pulmonary reflexes or various additional reflexes due to the contact between the victim’s body and drowning medium (DiMaio and DiMaio, 1993; Saukko, 2004; Bierens et al. 2016). Furthermore, some studies have shown that the penetration of liquid into the lungs occurs in almost all drowning cases, even those without macroscopic findings of liquid into the lungs (Bierens et al. 2016). Indeed, following the progressive hypoxia of the laryngeal muscles, the larynx generally relaxes, and the drowning medium is aspirated into the airway (Bierens et al. 2016). During this step, hypoxemia is also exacerbated by concomitant pulmonary oedema and the release of systemic catecholamines with subsequent vasoconstriction, pulmonary hypertension and right to left intra-pulmonary shunting (McEwen and Gerdin, 2016). In addition, water

into the lungs can determine systemic electrolyte disorders. Furthermore, during the drowning process, animals can swallow water with a consequent increased risk of vomiting, gastric content aspiration and systemic electrolyte disorder (McEwen and Gerdin, 2016; Bierens et al. 2016). Finally, the persistence of hypoxia causes convulsions and cardiological dysfunction, which can culminate in the death of the animal. Differences in systemic electrolyte disorder and in mechanism of lung injuries can be observed between freshwater and saltwater drowning. These differences are due to the difference in osmolarity between these two drowning mediums. Indeed, saltwater is hypertonic to the ion concentration of the lung cells and bloodstream. Therefore, plasma from the bloodstream enters the lungs to compensate for the differences in concentration. Thus, drowning in hypertonic fluids causes pulmonary oedema, damage to surfactant, haemoconcentration, erythrocyte haemolysis, intravascular potassium release and subsequent ventricular fibrillation (McEwen and Gerdin, 2016; Bierens et al. 2016). In contrast, freshwater is hypotonic compared with lung cells and bloodstream. Thus, haemodilution, hypervolaemia, dilution of and damage to pulmonary surfactant, atelectasis, decreased serum sodium, and hyperkalaemia can occur in dogs drowned in freshwater (McEwen and Gerdin, 2016; Bierens et al. 2016). However, in both cases, destruction of the normal integrity of the alveolar-capillary membrane can occur, and as a result, the blood plasma can enter in the alveoli with concomitant development of pulmonary oedema (Bierens et al. 2016).

2.1.2. Diagnosis of drowning

The diagnosis of drowning is reported in the literature as one of the most difficult in the field of human forensic pathology. Although drowning mechanisms and the associated injuries have been described extensively in the human medical literature, this diagnosis is still one of exclusion (McEwen and Gerdin, 2016). Indeed, a wide range of information is required to issue a diagnosis of drowning in human forensic pathology, such as information about the clinical history of the victim, crime scene analysis, testimony and physical evidence, microscopic and histological findings and ancillary test results (McEwen and Gerdin, 2016). In drowned bodies, the most common lesions reported in the literature are *pulmonary congestion, pulmonary oedema and pulmonary haemorrhage, stable froth in the trachea, mouth, or nasal passages, right ventricular distention and water into the stomach* (McEwen and Gerdin, 2016; Farrugia and Ludes, 2011). However, these injuries, although characteristic of drowning, are not conclusive because they can be detected during autopsies of cadavers who died for causes other than drowning. Similarly, the main histological injuries are nonspecific and include *intraalveolar haemorrhage, flooding of alveoli with pale or proteinaceous fluid, alveolar overdistension, attenuation of alveolar septa, aspirated stomach contents, bacteria or foreign material in alveoli or airways* (McEwen and Gerdin, 2016). Over the last year, many ancillary tests have been proposed for the diagnosis of drowning in human forensic pathology, such as *real-time PCR assays and electrolyte tests* (Shkrum and Ramsay, 2007; Byard and Summersiedes, 2011 Mc Ewen and Gerdin, 2016). However, among these, no one has received as much

attention as the diatom test. This test is currently considered the “gold standard” for the diagnosis of drowning in human forensic pathology (Piette and De Letter, 2006; Fucci et al. 2017). It is based on the theory that it is possible to detect diatoms in the organs of drowned victims if they aspirated water containing diatoms into the lung before death (Fucci et al. 2017). Diatoms are unicellular, photosynthetic and autotrophic organisms that live both in fresh water and saltwater. Usually, the size range of diatoms (between 20 and 200 μm) and their morphology allow them to percolate through the alveolo-capillary barrier and subsequently enter into the bloodstream during the pre-agonic state of drowning (Fucci et al. 2017; Verma et al, 2003; Lucci et al; 2008). Although the diatom test is a valid test to support the diagnosis of drowning, its sensitivity and specificity is still controversial; the main limitation is based on the potential postmortem contamination of the organs due to the ubiquitous nature of diatoms. Moreover, the possibility of false positive results due to ante-mortem penetration of diatoms in the bloodstream through the intestinal or respiratory tract has been proposed (Lunetta et al., 2013) Finally, negative diatom results have been reported in tissues of humans who died in drowning condition (Anand and Unmesh, 2016). In veterinary forensic pathology, the lack of medical literature, associated with the wide range of animals of interest, makes the diagnosis of drowning even more difficult than a drowning diagnosis for humans. In addition, although the use of the diatom test has been sporadically reported in veterinary medicine, to the best of the authors’ knowledge, no study has evaluated its sensitivity and specificity in veterinary contest (Fucci et al. 2017). Finally, only a few reports have investigated macroscopic and histological injuries in cases of

accidental and nonaccidental drowning in animals of veterinary forensic interest (McEwen, 2016). In light of these observations, the aims of this study are as follows: 1) to evaluate the macroscopic and microscopic findings in drowned animals and, specifically, to evaluate the contribution of necropsy and histological examination to determine the cause of death in drowning cases in veterinary forensic pathology; 2) to investigate the differences in the number and location of diatoms between animals who died in drowning and nondrowning conditions; and 3) to investigate the correlation between the time of permanence in water and the number and location of diatoms in animals dead for causes other than drowning and subsequently used for experimental drowning in standard conditions.

2.2. Materials and methods

2.2.1. Study design

Twenty-three dead adult animals were employed for the study and subdivided into six groups. Group A comprised 3 cadavers (1 lemur and 2 dogs) dead for drowning and recovered from aquatic environments; the group B comprised 5 control animals (4 dogs and 1 cat) dead for causes other than drowning; the group C comprised 5 animals (4 dogs and 1 cat) dead for causes other than drowning and subsequently immersed for 24 hours in water; the group D comprised 5 animals (4 dogs and 1 cat) dead for causes other than drowning and subsequently immersed for 48 hours in water. Finally, the group E comprised 5 animals (4 dogs and 1 cat) dead for causes other than drowning and subsequently immersed for 72 hours in water. All postmortem experimental drownings of the animals in groups B,

C and D were performed at room temperature (22 °C) using water from the pond of the zoological garden of Naples. In these cases, intermittent movement of the water was performed to ensure adequate interaction between the diatom-rich water and the submerged animals.

2.2.2. Water samples

Between 13 January and 22 April, 2018, samples of water were obtained from the three sites where the drowned animals (group A) were recovered: 1) the Mediterranean Sea in Agropoli and the Mediterranean Sea in Naples, where the two dogs were recovered, and the pond of the zoological garden of Naples, where the lemur was recovered. The samples were collected from the seashore and close to the banks of the lake using plastic bottles previously checked to verify the absence of diatoms. The water samples were used for diatomological mapping of the three sites and to assess the density of diatoms. In addition, water from the pond of the zoological garden of Naples was used for the postmortem drowning experiments.

2.2.3. Macroscopic examination

Postmortem examination was performed in all cadavers. All forensic necropsies were performed in the necropsy room of the Department of Veterinary Medicine and Animal Production of the University of Naples "Federico II" with a standard necropsy protocol previously described by Piegari et al. (2018).

2.2.4. Histological examination

Representative lung samples from groups A, B and C were collected for histopathologic examination; samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 microns and stained with haematoxylin and eosin (HE) for morphological evaluation of lesions (Piegari et al. 2018).

2.2.5. Diatom test

According to the protocol previously described by Fucci et al. (2017), for all cadavers, five grams of lung, liver, kidney, brain, spleen and bone marrow, and 10 ml of the drowning medium were processed for diatom test performed with the hydrogen peroxide digestion method. For the purposes of this study, we used the hydrogen peroxide method to better preserve the diatoms during the extraction process. Furthermore, to avoid contamination, an internal protocol was used to take the samples and to process them in the laboratory. This internal protocol included the use of cleaned and separate instruments for each sample and the use of Diatom-free water for washing. Furthermore, the use of cellulose wadding, chalked gloves, tap water and any nondiatom-free instrument was avoided. Tab. 1 summarizes the diatom test steps and the protocol used to avoid diatom contamination

Tab 1. diatom test protocol and internal protocol used to avoid diatom contamination

Diatom test step	Internal protocol to avoid diatom contamination
Necropsy	Sterile blades, change the blade after skinning to avoid skin contact with organs
Sample collection	Sterile blade, separate instruments for each sample, diatom-free glass containers
Samples treated with HCL (20%) for 24 hours	Sterile blade, separate instruments for each sample, diatom-free glass containers
Washing	Diatom-free water for washing
Samples treated with H ₂ O ₂ (40%)	diatom-free glass containers
Overnight sedimentation and decanting of excess liquid	//
Sediment centrifuged three times at 1000 rpm and washed	Diatom-free water for washing, diatom-free glass container

2.2.6. Diatom quantification and statistical analysis

After hydrogen peroxide digestion method, 300- μ l of pellet was transferred onto microscope slides $\pm 76 \times 26$ mm allowed to dry on five serially marked microscopic slides, and then mounted permanently with Naphrax resin (Brunel Microscopes Ltd., Chippenham, UK). Diatoms were examined using an optical microscope fitted with a high definition camera (Nikon Eclipse E1000, Tokyo, Japan) at 40x magnification for diatom counting and subsequent identification, both in the water samples and tissue samples. Diatoms were identified and classified according to the available literature.

The SPSS 20.0 package (SPSS Inc., Chicago, IL, USA) was used for statistical analysis of the data. The Mann–Whitney test, a nonparametric test for two independent samples, was used to assess the differences in diatom number between groups. P values less than 0.05 were considered statistically significant.

2.3. Results

2.3.1. Forensic necropsy

In all animals in group A, we observed mild to moderate pulmonary congestion and moderate to severe pulmonary oedema and pulmonary emphysema. Furthermore, in 1 case, we found water in the stomach, pulmonary haemorrhages and right ventricular distention. Finally, in 1 case, fracture of the ribs and subcutis haemorrhages were also observed. However, similar lesions were observed in 4 out of 5 cases in group B, 4 out of 5 cases in group C and in all cases in groups D and E (Tab. 2).

Drowning in veterinary forensic pathology

Tab. 2 Macroscopic Lesions of Drowning observed in study animals stratified by group

	Wet Hair coat	Pulmonary congestion	Pulmonary edema	Pulmonary hemorrhages	Pulmonary emphysema	Right ventricular distension	Water and or foreign material in stomach	traumatic lesions
Group A Lemur	X	X	X	X	X		X	
Group A Dog	X	X	X	X		X		
Group A Dog	X	X	X	X	X			X
Group B Dog								X
Group B Dog		X	X					
Group B Cat		X	X		X	X		
Group B Dog	X	X	X	X	X			X
Group B Dog	X	X	X	X				X
Group C Dog	X	X	X					
Group C Dog	X	X	X	X	X	X		X
Group C Dog	X	X	X			X		
Group C Dog	X	X	X	X	X	X		
Group C cat	X							X
Group D Dog	X	X	X			X		
Group D Dog	X	X	X	X	X	X		
Group D Dog	X	X	X		X	X		
Group D Cat	X	X	X			X		
Group D Cat	X	X	X	X	X			
Group E Dog	X	X	X	X	X	X		
Group E Dog	X	X	X		X	X		
Group E Cat	X	X	X		X	X		
Group E Dog	X	X	X	X	X	X		
Group E Dog	X	X	X		X	X		

2.3.2. *Histological analysis*

The histological examination performed on lung samples of the animals in group A revealed multifocal intra-alveolar haemorrhages, moderate to severe pale or proteinaceous fluid within alveoli, mild to moderate vasodilatation and rupture of several alveolar walls. Moreover, a mild interalveolar infiltrate of macrophages was also observed in 1 out of 3 cases. In addition, in the lemur lung, we observed foreign material in alveolar spaces, which, by morphology and location, could be compatible with diatoms or other algae. However, similar injuries were observed in the lungs of animals in groups B and C. In 4 animals in group B, we found multifocal haemorrhages and pulmonary oedema. In 2 out of 5 cases, we also observed mild to moderate vascular congestion and mild infiltration of neutrophil granulocytes and macrophages. Similarly, in all animals in group C, we observed moderate to severe intra-alveolar oedema associated with vascular congestion and, in 2 out of 5 cases, intra-alveolar haemorrhages (fig. 1).

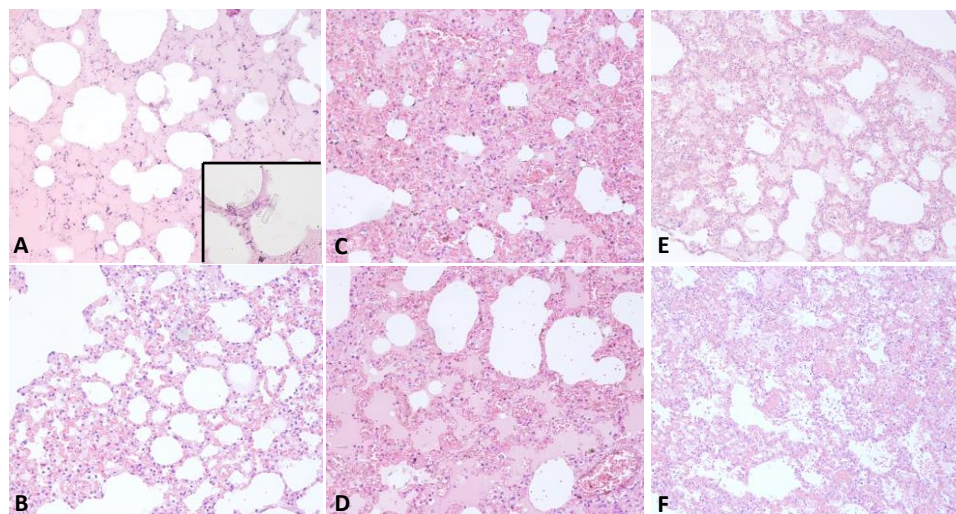


Figure 1. Representative H&E –stained sections from lung tissues of animals of the groups A, B and C. A: lung section from drowned animals showing proteinaceous fluid within alveoli. Inset: foreign material in alveolar space B: lung section from downed animals showing mild to moderate vasodilatation and emphysema. C-D: lung section from animals of the group B showing proteinaceous fluid in alveolar space, vasodilatation and emphysema. E-F: lung section from animals of the Group C showing proteinaceous fluid within alveoli, vasodilatation and intra-alveolar hemorrhages. Stain: H&E. original magnification 20x

2.3.3. Diatoms in Drowning medium

The range of diatoms per 300 μ l of pellet were 200-298 (mean: 242 +- 45 SD), 92-149 (mean: 115,25 +- 24 SD) and 60-111 (mean: 89 +- 21,67 SD) for the lake in the zoological garden of Naples, the Mediterranean Sea in Naples and the Mediterranean Sea in Pozzuoli, respectively. Sea samples contained characteristic centric and pennate diatoms, predominantly *Nitzschia* sp, *Navicula* sp and *Cymbella* sp. Furthermore, lake diatoms consisted predominantly of *Cyclotella* sp., *Cylindrotheca fusiformis* and *Bacillaria paxillifera*.

2.3.4. Diatoms in drowned and control animals

Different diatom diffusion was observed between drowned and control animals. In particular, diatoms were detected in the lung, liver and spleen of all animals in group A. Furthermore, diatoms were observed in the lemur brain tissues. With regard to the animals in group B (control animal), no diatoms were detected in 4 out of 5 cases, while in one case, diatoms were recovered from spleen and liver tissue samples. The most common diatoms detected in the animals in group A were *Cyclotella* sp., *Cylindrotheca fusiformis*, *Bacillaria paxillifera*, *Navicula* sp. and *Nitzschia* (fig. 2). Furthermore, all diatoms recovered from control animal tissue samples belonged to the genus *Naviculata*.

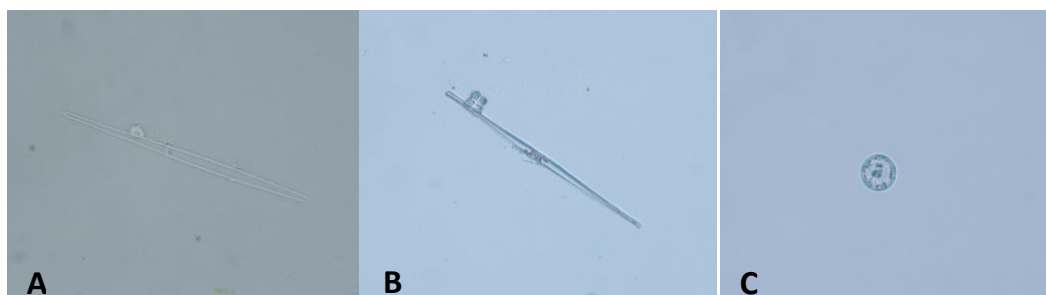


Figure 2. **The most common diatoms identified in animals of the Group A.** A-B: *Cylindrotheca fusiformis*; C: *Cyclotella* sp.

We observed differences in diatom number between drowned and control animals. Overall, the number of diatoms in each assessed organ of the drowned animals ranged from 0 to 79 frustules/300 μ l, while those in control animals ranged from 0 to 5. In addition, among drowned animals, we observed higher diatom numbers in lung tissue samples than those detected in other organs. Finally, the Mann-Whitney test showed differences in

diatom numbers between the drowned (Group A) and control group (Group B) (tab. 3). In particular, we observed significantly lower diatom numbers in the lung, liver, kidney and spleen of the control group (Group B) compared to the drowned animals (Group A). Table 4 summarizes the diatom numbers per 300 μ l recovered from group A and B animals stratified by assessed organ.

Tab 3. Mann–Whitney rank sum test between control and drowned animals

Animals	Lung	Liver	Kidney	Brain	Spleen	Bone marrow
Median (group A)	27	5	3	0	3	0
Median (group B)	0	0	0	0	0	0
P value	<0.05	<0.05	<0.05	=527	<0.05	=527

Tab 4. No. of diatoms per 300 μ l recovered from animal tissue samples

Animals	Lung	Liver	Kidney	Brain	Spleen	Bone marrow
Group A Dog	27	4	2	0	3	0
Group A Dog	22	4	2	0	3	0
Group A Lemur	79	5	3	3	8	4
Group B Dog	0	0	0	0	0	0
Group B Dog	0	5	0	0	3	0
Group B Cat	0	0	0	0	0	0
Group B Dog	0	0	0	0	0	0
Group B Dog	0	0	0	0	0	0

2.3.5. *Postmortem diatom penetration*

Diatoms were detected in the lungs of all experimentally drowned cadavers (group C= 24 h in water; group D= 48 h in water; and group E= 72 h in water). In addition, diatoms were recovered from liver and spleen tissue samples of 1 animal in group E (72 h in water). Overall, the number of diatoms recovered from lung tissue samples from group C animals ranged from 2 to 4 frustules/300 μ l, while those of groups D and E ranged from 2 to 5 and 6 to 10, respectively (tab. 5). The Mann-Whitney test showed no differences in diatoms number between group C and D lung tissue samples. However, the same test demonstrated differences in diatoms number between animals of groups C and E and groups D and E (tab. 6), between the experimentally drowned groups and drowned animals and between the control and experimentally drowned groups. In particular, we observed that the diatoms number recovered from lung tissue samples from group E were significantly higher than those observed from animals in groups C and D ($p < 0.05$). Furthermore, we found that the diatom numbers in the lung tissue samples from the experimentally drowned cadavers were significantly lower than those observed in drowned animals and significantly higher than those detected in the control group (Group B) ($p < 0.05$).

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Tab 5. No. of diatoms per 300 µl recovered from animal tissue samples

Animals	Lung	Liver	Kidney	Brain	Spleen	Bone marrow
Group C Dog	4	0	0	0	0	0
Group C Dog	3	0	0	0	0	0
Group C Cat	2	0	0	0	0	0
Group C Dog	4	0	0	0	0	0
Group C Dog	4	0	0	0	0	0
Group D Cat	3	0	0	0	0	0
Group D Dog	3	0	0	0	0	0
Group D Dog	3	0	0	0	0	0
Group D Dog	5	0	0	0	0	0
Group D Dog	2	0	0	0	0	0
Group E Cat	6	0	0	0	0	0
Group E Dog	9	4	0	0	1	0
Group E Dog	8	0	0	0	0	0
Group E Dog	8	0	0	0	0	0
Group E Dog	10	0	0	0	0	0

Tab 6. Mann–Whitney rank sum test of diatom test results in lung

Animals	P value
Group C vs D	>0.05
Group C vs E	<0.05
Group D vs E	<0.05

2.4. Discussion

The diagnosis of drowning is described in the literature as one of the most difficult in the field of human forensic pathology. Indeed, although the macroscopic and histological injuries of drowning have been described extensively in the human medical literature, this diagnosis is still one of exclusion (Mc Ewen and Gerdin, 2016). The most common injuries observed in drowning cases are located at the respiratory and cardiac level and include *pulmonary congestion, pulmonary oedema and pulmonary haemorrhages, lung distention, subpleural bullae of emphysema, sometimes haemorrhagic, stable froth in the trachea, mouth, or nasal passages and right ventricular distention* (Farugia et al., 2011, Pollanen, 1998, Szpilman et al., 2011; Lindstedt and Shaeffer, 2011; Munro R and Munro HJ, 2013; Mc Ewen and Gerdin, 2016). Similarly, in our study, the macroscopic and histological examinations of the animals in group A allowed us to observe the same classical injuries of drowning previously described in the human and veterinary literature, such as *pulmonary congestion, pulmonary oedema and right ventricular distention* (Mc Ewen and Gerdin, 2016). Interestingly, similar injuries were also observed in the control and experimentally drowned animals. These findings suggested a low contribution of necropsy and histological techniques in determining the correct cause of death in drowning cases. Indeed, as highlighted frequently in human forensic pathology, these injuries, although characteristic of drowning, are not conclusive because they can be detected during autopsies of cadavers dead for causes other than drowning (Farugia and Ludes, 2011). For example, pulmonary oedema or pulmonary haemorrhage as well as right ventricular

distention can be observed in a broad range of diseases, *such as allergic reactions, neurological diseases, acute kidney diseases or hearth diseases* (Sureka et al. 2015).

Regarding the analysis of water samples, our study showed a larger number of diatoms recovered in the lake compared to the sea. These differences could be attributed not only to the general differences in diatom density between drowning mediums but also to specific differences in diatom density related to sampling location and to lower resistance of the siliceous cell walls of sea diatoms compared to lake diatoms (Sidari et al; 1999). In relation to the diatoms recovered from animal tissues, we observed a statistically higher diatom number in the tissues of drowned animals than in the tissue of control animals. In particular, the number of diatoms in drowned animals ranged from 0 to 79 frustules/300 μ l, while those in control animals ranged from 0 to 5. In addition, we observed differences between control (Group B) and drowned animals (Group A) regarding diatom location. Indeed, diatoms were detected in the lung, liver and spleen of all animals in group A. Furthermore, diatoms were recovered from brain tissue samples of 1 animal in group A. Among the assessed organs, the highest diatom number was observed in lung tissue samples, with a range from 22 to 79 frustules/300 μ l. In contrast, in animals in group B (control animal), no diatoms were detected in 4 out of 5 cases, while in one case, few diatoms were recovered from liver and spleen tissue samples. Finally, all diatoms detected in the animals in group A matched with the respective drowning medium. The positive diatom results observed in the drowned animals could be explained by antemortem penetration of water containing diatoms into the lungs (MCEwein and Gerdin, 2016; Fucci et al, 2017). In these cases,

the small size range of diatoms and their morphology allow easy percolation through the alveolo-capillary barrier with subsequent entry into the bloodstream and diffusion to peripheral organs during the preagonic state of drowning (McEwein and Gerdin, 2016; Fucci et al, 2017). In contrast, the positive results observed in control animals could be explained by antemortem diatoms penetrating through the gastro-enteric system as a result of ingestion of diatom-rich food or water (Timperman et al.; Hendey, 1987; Yen and Jayaprakash, 2007). Moreover, the inhalation of diatoms should be considered a mechanism of diatom penetration in living animals (Koseki, 1968; Spitz and Schmndt, 1965). The possibility of false positive diatom results has been previously reported in human forensic pathology (Lunetta et. al, 2013). However, the human literature on antemortem diatom penetration is highly controversial. Foged et al. (1983), in a study conducted on 4 nondrowned human bodies, reported up to 194 valves/cm in lung, up to 54 valves/cm in liver and up to 17 valves/cm in bone marrow. However, the most recent studies conducted with internal protocols to avoid postmortem diatom contamination showed results strikingly in contrast than those obtained by Foged et al. (2018). For example, Lunetta et al. (2013), in a study conducted on 14 nondrowned bodies, detected only a few diatoms in 6 out of 14 cases. In contrast, Auer and Möttönen (1988) reported no diatoms recovered from internal organs of 15 nondrowned cadavers. Similarly, Bortolotti et al. (2011) showed the absence of diatoms in both lung and sternum samples of 45 nondrowned cadavers. In contrast, in veterinary forensic pathology, Fucci et al. (2017), in a recent study conducted on 10 drowned and nondrowned animals, showed a high number of diatoms recovered from tissue samples other than lungs, in otters who

died for causes other than drowning. Regarding postmortem diatom penetration in the experimentally drowned cadavers, diatoms were detected in the lungs of all animals in groups C, D and E. In addition, diatoms were recovered from liver and spleen tissue samples from 1 animal in group E (72 h in water). Furthermore, the Mann-Whitney test showed no differences in diatom numbers between lung tissue samples from groups C and E. The same test demonstrated differences in diatom numbers between lung tissue samples from groups C and E and groups D and E. In addition, in all experimentally drowned groups, the number of lung diatoms ranged from 2 to 10 frustules/300 μ l and was statistically lower than those observed in drowned animals (group A) and statistically higher than those detected in control animals (group B). Overall, the positive diatom results observed in the lungs of experimentally drowned animals could be explained by postmortem passive and gravity-dependent movement of water into the lung. In these cases, the absence of cardiac function should prevent diatoms from spreading to other tissues. However, the higher number of diatoms in the lungs of the group E animals compared to those of other experimentally drowned groups and the positive diatoms results observed in liver and spleen tissue samples of one animal in group E suggest a specific relationship between postmortem diatom diffusion and time of permanence in water. Indeed, postmortem changes, such as putrefaction and autolysis, play an important role in the postmortem diffusion of diatoms into cadavers. Diatoms can resist to the putrefaction. Therefore, with increasing cadaver decomposition, diatoms can spread into the lungs and enter into the other internal organs of the corpse, miming an antemortem spread due to drowning. Our results are in apparent agreement with those reported by Di

Giancampillo et al. (2010) that showed the presence of diatoms in heart, lungs and skin samples of pigs immersed in water for 1 month after death. However, the different assessed species and different times of permanence in water make these findings not directly comparable with the results reported in our study. Finally, the Mann-Whitney test showed differences between animals in group A and all experimentally drowned groups and control animals regarding diatom numbers recovered from lung tissue samples. Therefore, these findings suggest that the number of diatoms may be used as a valid tool to differentiate animals who died in drowning and nondrowning conditions, even if the latter were found in an aquatic environment.

2.5. Conclusion

The diagnosis of drowning is a challenge in human forensic pathology as well as in veterinary forensic pathology. Our results suggest that the diatom test is a valid tool to support the diagnosis of drowning in veterinary forensic pathology. In contrast, the histological and anatomopathological findings in drowning cases are less specific because they can be observed in a wide range of causes other than drowning. Our results showed differences in diatom density and location between drowned and nondrowned cadavers. Therefore, our study provides a reference basis to better establish separation values between drowned and nondrowned animals in veterinary forensic pathology.

- Anand TP, Unmesh A K, 2016. Diatom test: a reliable tool to assess death by drowning? *Int J Res Med Sci*, May;4(5):1479-1484
- Auer A, Möttönen M, 1988. Diatoms and drowning. *Z Rechtsmed*, 101:87–98.
- Badu IK, Girela E, Beltrán CM, Ruz-Caracuel I, Jimena I, 2015. Diatoms in forensic analysis: A practical approach in rats. *Med Sci Law*, Jul;55(3):228-35.
- Banting FG, Hall GE, Janes JM, Leibel B, Lougheed DW, 1938. Physiological studies in experimental drowning (a preliminary report). *Can Med Assoc*,39(3): 226–228.
- Beeck EF Van, Branche CM, Szpilman D, Modell JH, Bierens JJ, 2005. A new definition of drowning: towards documentation and prevention of a global public health problem. *World Heal Organ*, 83(5):853–856.
- Bhardwaj W, Mohan M, Rai UC, 1982. Electrocardiographic changes in experimental drowning. *Indian J Physiol Pharmacol*, Jan-Mar;26(1):85–89.
- Bierens JJ, Lunetta P, Tipton M, 2016. Warner DS4. *Physiology* (Bethesda), Mar;31(2):147-66.
- Bortolotti F, Del Balzo G, Calza R, Valerio F, Tagliaro F, 2011. Testing the specificity of the diatom test: search for false-positives. *Med Sci Law*, 51 Suppl 1:S7-S10.
- Byard RW, Summersides G, 2011. Vitreous humor sodium levels in immersion deaths. *J Forensic Sci*, May;56(3):643–644.
- Conn AW, Miyasaka K, Katayama M, et al., 1995. A canine study of cold water drowning in fresh versus salt water. *Crit Care Med*, Dec;23(12):2029–2037.

Di Giancamillo A, Giudici E, Andreola S, Porta D, Gibelli D, Domeneghini C, Grandi M, Cattaneo C (2010). Immersion of piglet carcasses in water--the applicability of microscopic analysis and limits of diatom testing on an animal model. *Leg Med (Tokyo)*, Jan;12(1):13-8.

DiMaio DJ , DiMaio VJM, 1993 .Forensic Pathology, Baco Raton, FL
CRC Press

Farrugia A and Ludes B (2011). Diagnostic of Drowning in Forensic Medicine, *Forensic Medicine - From Old Problems to New Challenges*, Prof. Duarte Nuno Vieira (Ed), InTech, Available from:
<http://www.intechopen.com/books/forensic-medicine-from-old-problems-to-new-challenges/diagnostic-of-drowning-in-forensic-medicine>

Foged N, 1983. Diatoms and drowning-once more. *Forensic Sci Int*, Mar-Apr;21(2):153–9.

Fucci N, Campobasso CP, Mastrogiuseppe L, Puccinelli C, Marcheggiani S, Mancini L, Marino L, Pascali VL, 2017. Diatoms in drowning cases in forensic veterinary context: a preliminary study. *Int J Legal Med*, Nov;131(6):1573-1580.

Hendey NI, 1973. The diagnostic value of diatoms in cases of drowning. *Med Sci Law*, Jan;13(1):23–34.

Idris AH, Berg RA, Bierens J, et al. 2003. Recommended guidelines for uniform reporting of data from drowning the “Utstein style.” *Resuscitation*, Oct;59(1):45-57.

Layon AJ, Modell JH, 2009. Drowning update. *Anesthesiology*, Jun;110(6):1390-401.

- Lucci A, Campobasso CP, Cirnelli A et al, 2008. A promising microbiological test for the diagnosis of drowning. *Forensic Sci Int*, Nov 20;182(1-3):20-6
- Lunetta P, Modell JH. Macroscopical, microscopical, and laboratory findings in drowning victims: a comprehensive review. In: Tsokos M, ed. *Forensic Pathology Reviews*. Totowa, NJ: Humana Press; 2005:3–77
- Lunetta P , Miettinen A, Spilling K, Sajantila A, 2013. False-positive diatom test: A real challenge? A post-mortem study using standardized protocols. *Leg Med (Tokyo)*. Sep;15(5):229-34.
- Lindstedt SL, Schaeffer PJ, 2002. Use of allometry in predicting anatomical and physiological parameters of mammals. *Lab Anim*, Jan;36(1):1-19.
- McEwen BJ, Gerdin J, 2016. *Veterinary Forensic Pathology: Drowning and Bodies Recovered From Water Veterinary Pathology*. *Vet path*, Sep;53(5):1049-56.
- Modell JH , Bellefleur M , Davis JH, 1999. Drowning without aspiration: is this an appropriate diagnosis?. *J Forensic Sci*, Nov;44(6):1119-23.
- Munro R, Munro HJ, 2013. Some challenges in forensic veterinary pathology: a review. *Comp Pathol*, Jul;149(1):57-73.
- Piette MH, De Letter EA, 2006. Drowning: still a difficult autopsy diagnosis. *Forensic Sci Int*, Nov 10;163(1-2):1-9.
- Pollanen MS, 1998. Diatoms and homicide. *Forensic Sci Int*, Jan 9;91(1):29-34.
- Saukko P, 2004. *Knight Immersion deaths B. Chapter 16 Knight's Forensic Pathology* Saukko P , Knight B London, UK Arnold Publishing.

- Shkrum M, Ramsay D, 2007. *Forensic Pathology of Trauma: Common Problems for the Pathologist*. Totowa, NJ: Humana Press.
- Sidari L, Di Nunno N, Costantinides F, et al., 1999. Diatom test with Soluene-350 to diagnose drowning in sea water. *Forensic Sci Int*, Jul 12;103(1):61-5.
- Spitz WU, Schmidt H, Fett W, 1965. Studies on air filtration strips from different regions of the federal republic for their diatom content. A contribution to the value of diatoms in the diagnosis of death by drowning. *Dtsch Z Gesamte Gerichtl Med*, Jun 8;56:116-24.
- Sureka B, Bansal K, Arora A, 2015. Pulmonary edema – cardiogenic or noncardiogenic?. *J Family Med Prim Care*, Apr-Jun; 4(2): 290
- Szpilman D, Bierens JJLM, Handley AJ, Orlowski JP, 2012. Drowning. *N Engl J Med*, May 366(22):2102–2110
- Timperman J, 1969. Medico-legal problems in death by drowning. Its diagnosis by the diatom method. A study based on investigations carried out in Ghent over a period of 10 years. *J Forensic Med*, Apr-Jun;16(2):45-75.
- Verma K, 2013. Role of diatoms in the world of forensic sciences. *J Forensic Res*, May-Jun 4:2–4
- Koseki T, 1968. Fundamental examinations of experimental materials and control animals on the diagnosis of death from drowning by the diatom method. *Acta Med Biol*, Jun;15:207–19.
- Yen LY, Jayaprakash PT, 2007. Prevalence of diatom frustules in non-vegetarian foodstuffs and its implications in interpreting identification of diatom frustules in drowning cases. *Forensic Sci Int*, Jul 20;170(1):1-7

Forensic thanatology

Chapter 3

Evaluation of Muscular Proteins Degradation to Define Post Mortem Interval (PMI) in Dogs

BASED ON: Giuseppe Piegari, Rosario Fico, Alessandro Costagliola, Davide De Biase, Francesco Prisco, Serenella Papparella, Orlando Paciello. Evaluation of Muscular Proteins Degradation to Define Post Mortem Interval (PMI) in Dogs; Sisvet meeting, 2017

3.1.Introduction

In general, almost immediately after death, an irreversible and progressive sequence of physical and biochemical changes occurs in the body of cadavers. Normally, these changes have a well-defined order of progression; however, the rate of their onset and development is influenced by a wide range of variables that are both environmental and intrinsic to the subject itself, such as *weight, age, size, amount of adipose tissue, previous diseases and state of health of the animal before death, environment temperature, humidity and air flow*. A knowledge of the rate of development of postmortem changes is useful not only to better interpret the macroscopic and histological findings at necropsy but also to estimate the time since death, also called the “postmortem interval (PMI)” (Brooks, 2018). The postmortem interval is important in human forensic pathology and is equally important in veterinary forensic pathology. Determining the PMI can in fact provide law enforcement officials with useful information to include or exclude particular individuals in a group of suspects or possibly validate the testimony of a witness (Brooks, 2016). Unfortunately, although studies on the postmortem interval are one of the most popular topics of research in human forensic medicine, in veterinary medicine, published data on postmortem changes and their rate of development are scarce (Brooks, 2016). In addition, to the best of our knowlagent, no study has investigated the post mortem histological and immunohistochemical modification of the skeletal muscle in dog. Overall, there are several reasons why skeletal muscle could be a promising candidate tissue for use in PMI delimitation in veterinary forensic pathology. First, it is the most abundant tissue of the

animal's body and it is relatively well protected by skin. Therefore, it should be less influenced by extrinsic variables than other organs such as eye. In addition, previously studies showed that muscle tissue has a much greater delay in postmortem changes compared to other organs such as liver or kidney (Vass et al, 2002). In the light of these observations, the aim of this study is to assess specific correlation between post-mortem histological changes of the muscle tissues and post-mortem degradation of the cytoskeletal proteins desmin and dystrophin with time since death in dog.

3.2. Material and methods

3.2.1. *Muscle samples collection*

Twenty-five dead dogs (10 male, 15 female) of various breeds, aged between 6 and 12 years of age, were recruited into the study. Animals causes of death were diverse and included 5 natural cases and 20 cases of euthanasia with informed owner consent by means of intravenous injection of 10 mg/kg Pentothal (Abbott Laboratories) and 70 mg/kg Tanax (MSD Animal Health). None of the studied dogs showed any clinical evidence of neuromuscular disease or metabolic diseases including hypothyroidism, hyperadrenocorticism, renal disease, diabetes mellitus or neoplasia. Each owner consented to use the cadaver for research purposes, according to the ethical guidelines of the Department of Veterinary Medicine and animals production of the University of Naples Federico II. Immediately after death, animals were maintained at standard temperature (23°C) for 4 days at the Section Room of the Department of Veterinary medicine of the University of Naples “Federico II”. For each animal, at 30 minutis and at 24, 48, 72, 96 hours after death, a cube of 1x1x1 cm of muscle tissue was removed from the vastus lateralis and triceps brachii. The samples were then divided into six groups on the basis of time elapsed from death:

- The Group A comprised 50 samples (25 samples from triceps brachii muscle and 25 samples from vastus lateralis muscle) taken 30 minutes after death of the animals.

- The Group B comprised 50 samples (25 samples from triceps brachii muscle and 25 samples from vastus lateralis muscle) taken 24h after death
- The Group C comprised 50 samples (25 samples from triceps brachii muscle and 25 samples from vastus lateralis muscle) taken 2 day after death
- the Group D comprised 50 samples (25 samples from triceps brachii muscle and 25 samples from vastus lateralis muscle) taken 3 days after death.
- the Group E comprised 50 samples (25 samples from triceps brachii muscle and 25 samples from vastus lateralis muscle) taken 4 days after death of the animals.

All samples were frozen in isopentane pre-cooled in liquid nitrogen, and stored at -80°C until further processed.

II.II Histological and Immunohistochemical examination

For histology, tissue sections of $10\ \mu\text{m}$ were cut in a transverse plane with a cryostat (-20°C) and stained with haematoxylin and eosin (H&E) stain.

Immunohistochemical analysis was performed according to the method previously described (Paciello and Papparella, 2009). Briefly, $10\ \mu\text{m}$ sections of muscle tissue were obtained using a cryostat. Slides were air dried for 1 h at room temperature, washed in phosphate-buffered saline (0.01 M PBS, pH 7.2) and fixed in acetone at 4°C for 3 min. Endogenous

peroxide activity was blocked using 0.3% hydrogen peroxide in methanol (Sigma–Aldrich), applied for 15 min at room temperature. After two washes in PBS, sections were incubated for 30 min at room temperature with Background Sniper (Biocare Medical). Sections were incubated overnight at 4 °C with the primary antibodies against Desmin (Santa Cruz Biotechnology) diluted 1:200 and Distrofin (Abcam), diluted 1:200;

After two washes with PBS, the MACH 1 Universal HPR-Polymer Detection Kit (Biocare Medical) was used according to the manufacturer's instructions. The sections were subsequently counterstained in haematoxylin, dehydrated in alcohol, clarified in xylene and mounted in aqueous mounting medium. For each section, 10 fields at 20x magnification were examined under light microscope separately by two independent pathologists (GP, OP) with a concordance rate of 95%.

The mean proportion of immunopositively cells was scored as follows: +++++ (>95% positively stained fibers in the section) +++++ (80-95% positively stained fibers in the section), +++ (50-80% positively stained fibers in the section) ++ (30-50% positively stained fibers in the section), + (1-30% positively stained fibers in the section) , - (negative staining observed in the fibers of the section). For statistical analysis, these values were transformed into numeric values, in which the highest possible rating “+++++” corresponded to 5 and the lowest rating “-” corresponded to 0.

3.2.2. *Statistical analysis*

The SPSS 20.0 package (SPSS Inc., Chicago, IL, USA) was used for statistical analysis of the data. The Kruskal–Wallis test, a non-parametric test for more than two independent samples, was used to compare groups. Also, specific contrasts for each assessed variable were carried out using the non-parametric Mann–Whitney test for two independent samples. P values less than 0.05 were considered statistically significant.

3.3. Results

3.3.1. *Histological examination*

Sections of muscle from the Group A (30 minutes after death) stained with H&E showed intact muscle fibers and no microscopic changes (fig. 1a) in both triceps brachii and vastus lateralis muscles. Samples from the group B (1 dpm) showed loss of cell border and small focal areas of autolysis (fig. 1b). In the samples from the Groups C (2 dpm), D (3 dpm) and E (4 dpm), we observed reduced fibre–fibre adhesion and small multifocal areas of autolysis with disintegration and ruptured of the fibres and loss of cell borders (fig. 1c, 1d, 1e).

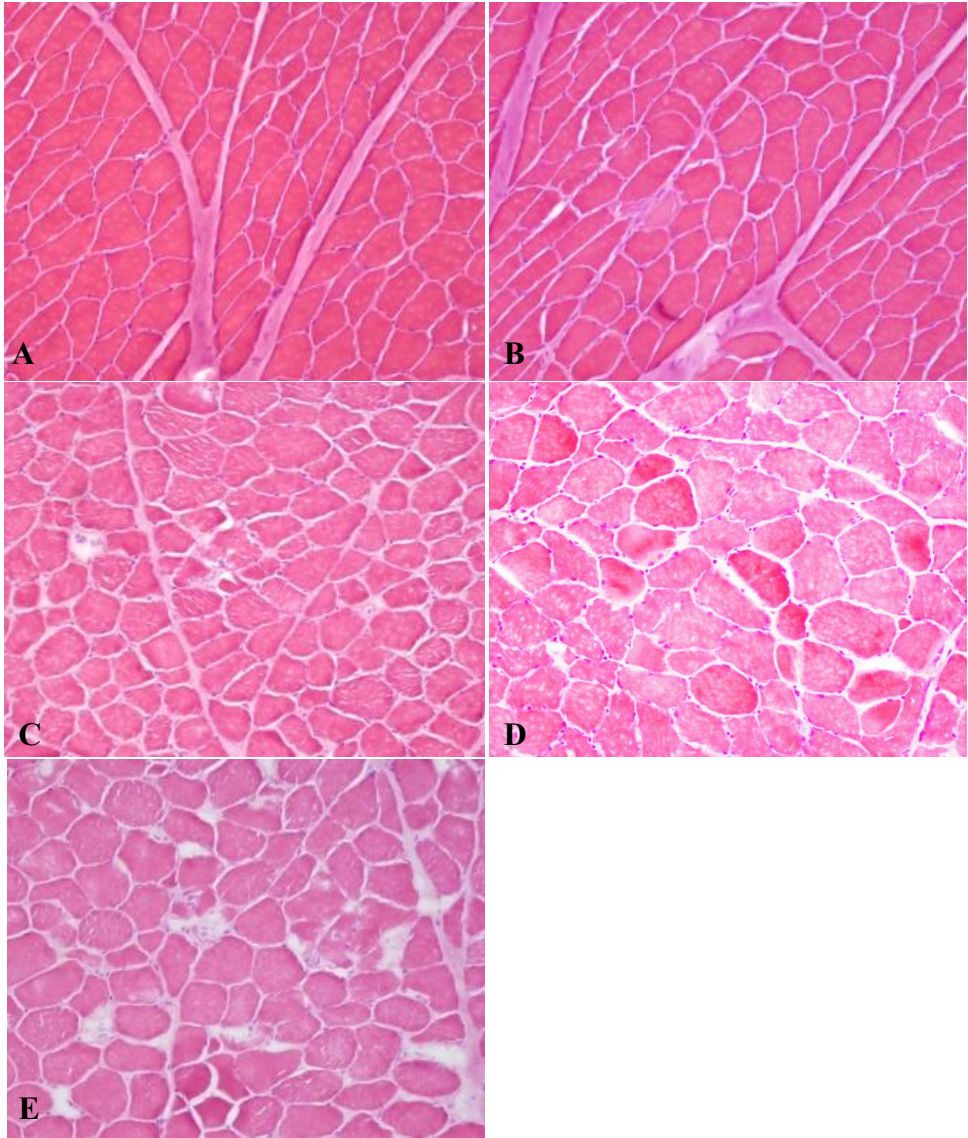


Figure 3. **Representative H&E –stained sections from muscle tissues at different time elapsed since death.** A: section from muscles taken at 30 minutes after death showing no microscopical changes; B: section from muscle taken at 1 day post mortem showing loss of cell border and focal areas of autolysis. C-D-E: section from muscles taken at 2, 3 and 4 days post mortem respectively. Note the reduced fibre–fibre adhesion and multifocal areas of autolysis with disintegration and ruptured of the fibres and loss of cell borders.

3.3.2. Immunohistochemical examination

In Muscles from the Group A (30 minutis after death), desmin and dystrophin immunoreactivity was observed in all assessed samples demonstrating >95% desmin and dystrophin positive fibers in both triceps brachi and vastus lateralis muscles . The muscle samples from the Group B (1 dpm) were positive on immunohistochemistry for desmine and dystrophin. However, the majority of tissue samples demonstrating 80-95% dystrophin (triceps brachii: 23/25 samples; vastus lateralis: 24/25 samples) and desmin (triceps brachii: 24/25 samples; vastus lateralis: 24/25 samples) positive fibers in the sections of both assessed muscles. As regard the muscles of the Group C (2 dpm), the majority of tissue samples demonstrating 50-80% dystrophin (triceps brachii: 22/25 samples; vastus lateralis: 23/25 samples) and desmin (triceps brachii: 24/25 samples; vastus lateralis: 24/25 samples) positive fibers in the section. The prevalence of desmin and dystrophin positive fibers in group D was as follows: 1-30% for dystrophin (triceps brachii: 25/25 samples; vastus lateralis: 24/25 samples) and 50-80% (triceps brachii: 21/25 samples; vastus lateralis: 22/25 samples) for desmin. Finally, in the group E (4 dpm), the majority of tissue samples showed no immunopositivity for dystrophin (triceps brachii: 25/25 samples; vastus lateralis: 24/25 samples) and 1-30% (triceps brachii: 21/25 samples; vastus lateralis: 21/25 samples) positive fibers for desmin (fig. 2 and 3; tab 1).

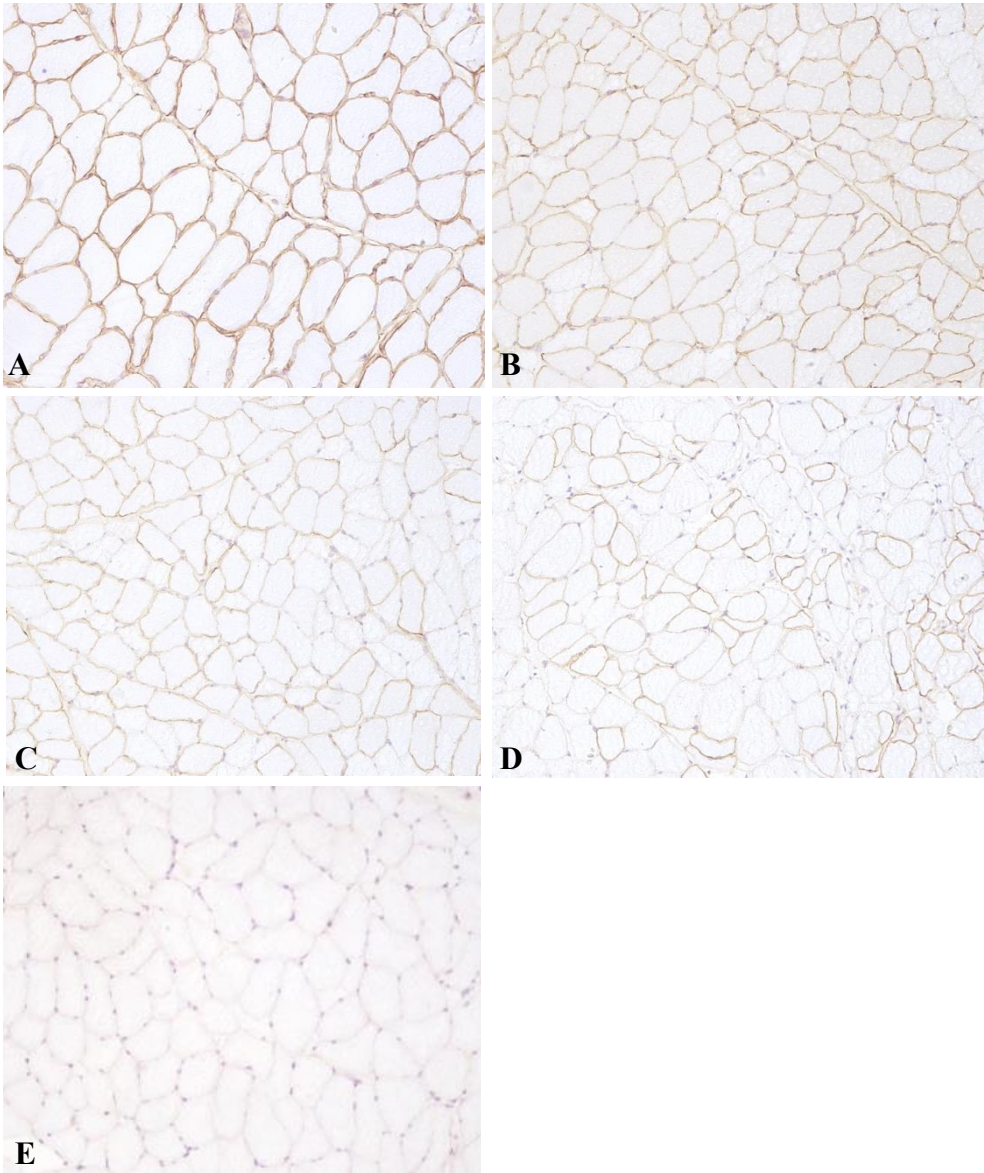


Figure 2. Changes produced in immunohistochemical labelling for dystrophin in cross-sections of dog muscles during post mortem storage. A: section from muscles taken at 30 minutis after death showing >95% dystrophin positive fibers; B: section from muscle taken at 1 day post mortem showing 80-95% dystrophin positive fibers in the section; C: section from muscles taken at 2 days post mortem showing 50-80% dystrophin positive fibers in the section. D: section from muscles taken at 3 days post mortem showing 1-30 % dystrophin positive fibers. E: section from muscles taken at 4 days post mortem showing no immunopositivity for dystrophin.

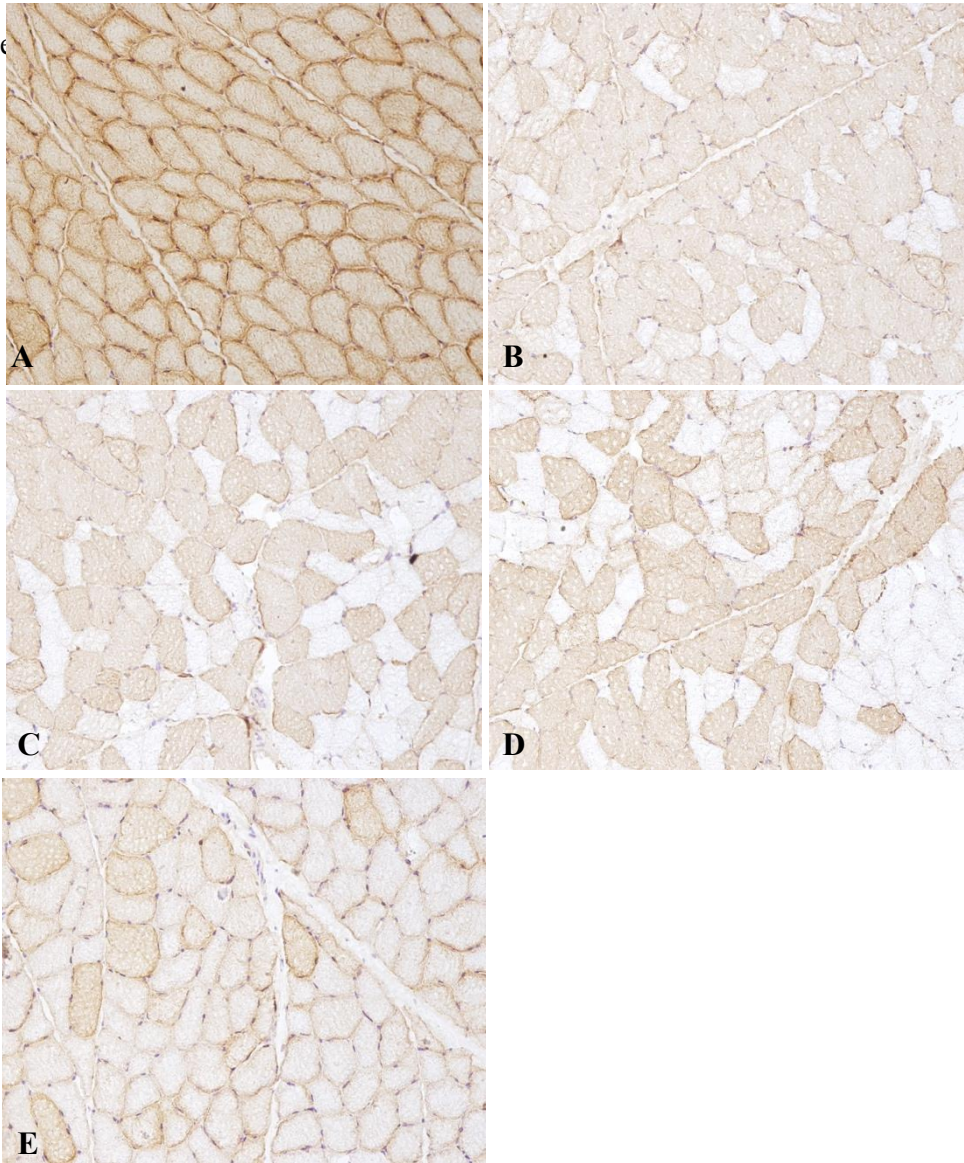


Figure 3. Changes produced in immunohistochemical labelling for desmin in cross-sections of dog muscles during post mortem storage. A: section from muscles taken at 30 minutis after death showing >95% desmin positive fibers; B: section from muscle taken at 1 day post mortem showing 80-95% desmin positive fibers in the section C: section from muscles taken at 2 days post mortem showing 50-80% dystrophin positive fibers in the section. D: section from muscles taken at 3 days post mortem showing 50-80 % desmin positive fibers. E: section from muscles taken at 4 days post mortem showing 1-30% positive fibers in the section.

Tab. 1 semi-quantitative observations on the muscle sections labelled with anti-desmin and anti-dystrophin primary antibodies

Groups	Dystrophin		Desmin	
	Trices brachii	Vastus lateralis	Trices Brachi	Vastus lateralis
Group A	+++++	+++++	+++++	+++++
Group B	++++	++++	++++	++++
Group C	+++	+++	+++	+++
Group D	+	+	+++	+++
Group E	-	-	++	++

Immunoreactivity: +++++, >95% of positive fibres in the section; +++++, 80-95%; +++ 50-80%; ++, 30-50%; +, 1-30%; -, negative

Statistical analysis showed differences in number of dystrophin positive fibers between Groups A and B, between Groups B and C, between Groups C and D and between Groups D and E in both assessed muscles (P<0.05). As regard the number of desmin positive fibres, statistical differences were observed between Groups A and B, between Groups B and C and between Groups D and E (p<0.05).

3.4. Discussion

Our study showed a specific correlation between histological and immunohistochemical changes in muscle tissue and time elapsed since death in dog. As regard the histological examination, we observed no microscopic modification in muscles taken at 30 minutes after death while, in muscles taken until 4 days post-mortem, we observed only small focal or multifocal areas of autolysis with disintegration and ruptured of the fibers and loss of cell borders. These results suggest a good post-mortem preservation of the muscle tissue in dog.

These findings were in agreement with those observed by Erlandsson (2007) that reported a good preservation of the dog myocardium until 7 days post-mortem. Similarly, the present results match with those reported by Tavichakortrakool et al. (2008) who detected histological changes, such as vacuolization and autolysis, in human muscles stored at 25 °C from 6h after death. Unfortunately, a direct comparison between aforementioned studies and our results is difficult due to the different storage temperatures which can inevitably influence the rate of development of the post-mortem changes in muscle tissue. As regard the immunohistochemical examination, in the majority of tissue samples, we observed a rapid post-mortem degradation of dystrophin with complete disappears of immunoreactivity towards anti-dystrophin antibody at 4 dpm. In addition, statistical analysis showed differences in number of dystrophin positive fibers between Groups A and B, between Groups B and C, between Groups C and D and between Groups D and E in both assessed muscles ($P < 0.05$). In contrast, as regard the desmin, most of tissue samples showed a slow post-mortem degradation

with a range of 1-30 % positive fibers in the section at 4 days post-mortem. In addition, statistical analysis showed differences in number of desmin positive fibres between groups A and B, between groups B and C and between groups D and E ($p < 0.05$). Over the last years, 3 different proteinases system have been studied to investigate their role in the post mortem proteolysis of the skeletal muscles: *the calpain system, the lysosomal cathepsins, and the multicatalytic proteinase complex (MCP)*. Studies conducted in vitro showed that the myofibrillar proteins are a poor substrates for multicatalytic proteinase complex (Koochmaraie, 1992). Thus, they can not be degraded by MPC during post-mortem storage of the muscles. In contrast, calpain system is reported in literature as the main responsible for the post-mortem degradation of the myofibrillar proteins, such as desmin and dystrophin, in animals (Geesink et al., 2006; Koochmaraie et al., 1986; Huff-Lonergan et al., 1996; Geesink and Koochmaraie, 1999). However, Calpastatin is the main inhibitor of the calpain proteinase (Mellgren, 1988). Therefore, its activity could influence the rate of post mortem myofibrillar degradation in muscles tissue. Indeed, overexpression of calpastatin in transgenic mice showed a reduction of the muscle proteins post-mortem proteolysis (Kent et al., 2004). Similarly, differences in calpastatin activity have been proposed as cause of the differences in the rate of postmortem muscle proteolysis between *Bos Taurus* and *Bos indicus* cattle. (Whipple et al., 1990); However, although calpain and the calpastatin activity play an important role in post-mortem proteolysis of both desmin and dystrophin, our findings identified a different rate of degradation between these two cytoskeletal proteins. Therefore, our data suggested that the simultaneous evaluation of desmin and dystrophin

could be used as a valid tool to estimate the time elapsed since death in both early (<3 days post-mortem) and intermediate (3-7 days post mortem) post-mortem phases.

3.5. Conclusion

The present study demonstrated that the post-mortem histological examination of the muscle tissue and the immunohistochemical analysis of muscle proteins degradation have a high potential for being a useful tool to assess the post mortem interval in veterinary forensic pathology. In the present study, we identified proteins with different rate of post-mortem degradation. Our findings suggested that the slow post-mortem degradation of the desmin makes it a valid marker to assess the intermediate (3-7 days) post-mortem phase while, the rapid post-mortem degradation of the dystrophin makes it a valid biomarker to assess the early (< 3 days) post-mortem phase in dog.

References

Brooks JW (ed.). *Veterinary Forensic Pathology*, Springer Verlag; 1st ed. 2018, ISBN-10: 3319671731

Brooks, JW, 2016. Postmortem changes in animal carcasses and estimation of the postmortem interval. *Vet Pathol*, Sep;53(5):929-40.

Erlandsson M, Munro R, 2007. Estimation of the post-mortem interval in beagle dogs. *Sci Justice*, Dec;47(4):150-4.

Geesink GH, Koohmaraie M, 1999. Postmortem proteolysis and calpain/calpastatin activity in callipyge and normal lamb biceps femoris during extended postmortem storage. *J Anim Sci*, Jun;77(6):1490-501.

Geesink GH, Kuchay S, Chishti AH, Koohmaraie M, 2006. Micro-calpain is essential for postmortem proteolysis of muscle proteins. *J Anim Sci*, Oct;84(10):2834-40.

Huff-Lonergan E, Mitsuhashi T, Beekman DD, Parrish FC, Olson DG, Robson RM, 1996. Proteolysis of specific muscle structural proteins by calpain at low pH and temperature is similar to degradation in postmortem bovine muscle. *J Anim Sci*, May;74(5):993-1008.

Koohmaraie M, 1992. Ovine skeletal muscle multicatalytic protein-ase complex (Proteasome): Purification, characterization, and comparison of its effect on myofibrils with-calpain. *J Anim.Sci*, Dec;70(12):3697-708.

Koohmaraie, M, Schollmeyer JE, Dutson TR, 1986. Effect of low-calcium-requiring calcium activated factor on myofibrils under varying pH and temperature conditions. *J Food Sci*, 51:28–32

Kent MP, Spencer MJ, Koohmaraie M, 2004. Postmortem proteolysis is reduced in transgenic mice over expressing calpastatin. *J Anim. Sci*, Mar;82(3):794-801.

Mellgren RL, 1988. On the mechanism of binding of calpastatin, the protein inhibitor of calpains, to biologic membranes. *Biochem Biophys Res Commun*, Jan 15;150(1):170-6.

Paciello O, Papparella S, 2009. Histochemical and immunohistological approach to comparative neuromuscular diseases, *Folia Histochem Cytobiol*, 47(2):143-52.

Tavichakorntrakool R, Prasongwattana V, Sriboonlue P, Puapairoj A, Pongskul J, Khuntikeo N, Hanpanich W, Yenichitsomanus PT, Wongkham C, Thongboonkerd V, 2008. Serial analyses of postmortem changes in human skeletal muscle: A case study of alterations in proteome profile, histology, electrolyte contents, water composition, and enzyme activity. *Proteomics Clin Appl*, Sep;2(9):1255-64.

Vass AA, Barshick SA, Sega G, Caton J, Skeen JT, Love JC, Synstelién JA, 2002. Decomposition chemistry of human remains: a new methodology for determining the postmortem interval. *J Forensic Sci*, May;47(3):542-53.

Whipple G, Koohmaraie M, Dikeman ME, Crouse JD, Hunt MC, Klemm RD, 1990. Evaluation of attributes that affect longissimus muscle tenderness in *Bos Taurus* and *Bos indicus* cattle. *J. Anim. Sci*, Sep;68(9):2716-28

Forensic traumatology

Chapter 4

Cardiac laceration following non-penetrating chest trauma in
dog and cat

Based on: G.Piegari, F. Prisco, D. De Biase, Fico R., Meormartino R., Paciello O. Cardiac laceration following non-penetrating chest trauma in dog and cat, 2018, Forensic Science International.

4.1.Introduction

Cardiac laceration with non-penetrating chest trauma (NCT) is reported as a common cause of death in human following rapid deceleration in high-speed vehicular accident (Modi et al., 2013; Brathwaite et al., 1990; Fulda et al., 1991). According to Getz et al. (1986), the involved mechanisms of the cardiac injury may be summarized as following: 1) direct blow to the anterior chest wall; 2) indirect injury with subsequent increased preload of the hearth; 3) compression of the heart between the sternum and vertebral bodies; 4) acceleration/deceleration of the heart; 5) blast forces; 6) penetrating injury due to the fractures of the ribs. In veterinary medicine, traumatic rupture of heart and great-vessel structures appears to be more rare than human and, to the authors knowledge, only rarely reported in literature (Harpster et al, 1974). Here we report the first case of cardiac rupture following NCT in a cat. Moreover, we present unusual cases of cardiac rupture with NCT in two dogs

4.2. Case report

4.2.1. Case 1

A 7-year-old, mixed, cat was found dead on the roadside and a complete necropsy was performed to find out the cause of death of the animal. The forensic examination was conducted in the necropsy room of the Department of Veterinary Medicine of the University of Naples “Federico II” following a standard necropsy protocol previously described by Piegari et al. (2018). The macroscopic examination revealed multifocal hemorrhages of the myocardium associated with a myocardial laceration of 0,5 cm on the lateral- inferior portion of the right ventricle (Fig. 1A); a total of 100 cc of clotted blood in the pericardial cavity but no tear of the pericardium was also observed. In addition, we found subcutaneous hemorrhages on the right thoracic region, fractures of the third and fourth rib and a contusion on the cranial lobe of the right lung. Representative samples of the heart were collected for the histopathologic examination; tissue was fixed in 10% neutral buffered formalin, paraffin embedded, sectioned at 4mm and stained with Hematoxylin and Eosin (HE). Histologically, we observed a hemorrhagic laceration of the heart associated with plurifocal hemorrhages between the myocardial fibres (Fig. 1B). Based on histological and macroscopic data, a definitive diagnosis of cardiac rupture was made. Finally, cardiac tamponade was considered as cause of death.

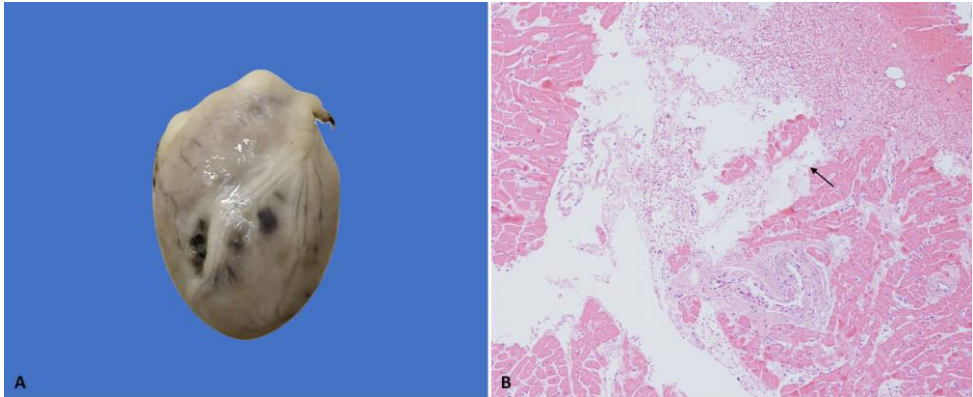


Figure 1: *cat. (A) cardiac laceration; tear on the lateral-inferior portion of right ventricle near inter-ventricular septum. (B) Histopathological section from margin of tear; hemorrhagic laceration of the heart (arrow); H&E stain (original magnification 20x)*

4.2.2. Case 2

A 13-year-old, mixed, dog was found dead on the roadside and a complete necropsy was performed to find out the cause of death. During the forensic examination we observed: myocardial and pericardial injury associated with 450 cc of clotted blood in the thoracic cavity. Large vertical tear of 3 cm was identified on the upper third of the pericardium (Fig. 2A) and, within the pericardial sac, only a little fragment of the right ventricle of 3x2 cm size was found (Fig. 2B); fragments of the heart were also identified in the chest cavity mixed with the clotted blood. In addition, we observed: contusion on the caudal lobe of the left lung, subcutaneous hemorrhages on the left thoracic region, fractures of the skull as well as fractures of the second, third and fifth right rib. Based on the macroscopic examination, “hypovolemic shock”, due to severe cardiac rupture, was considered as cause of death.

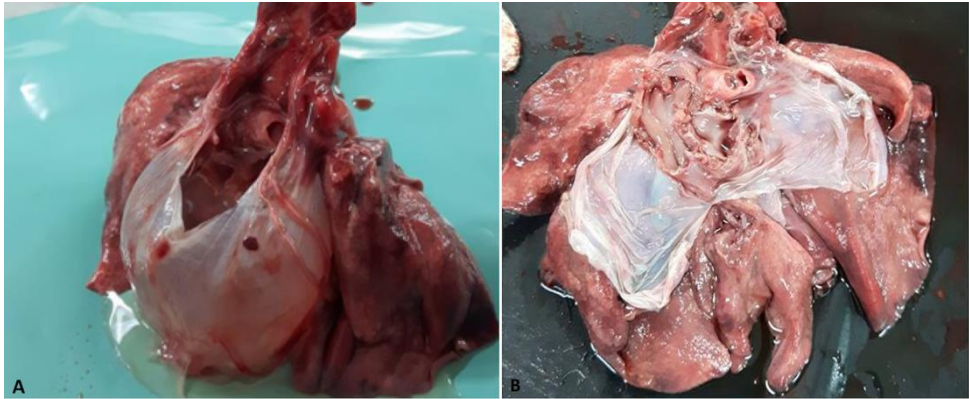


Figure 2: dog. macroscopic examination: (A) pericardium filled with water shows a large vertical tear on its upper third (B) little fragment of the right ventricle within the pericardial sac.

4.2.3. Case 3

A 2-year-old, mixed, dog was found dead on the roadside and a complete necropsy was performed to find out the cause of death of the animal. The macroscopic examination did not reveal any external chest injuries. However, in the thoracic cavity, we observed pericardial tear associated with a laceration of size 0.6 cm x 0.4 cm over the right auricle of the heart (Fig. 3B); a total of 150 cc of clotted blood in the thoracic cavity was also observed (Fig. 3A). In addition, we found multifocal hemorrhages of the lung and a focal peri-aortic hemorrhage of size 1.4x2.0 cm. Representative samples of the heart and aorta were collected for the histopathological examination; tissues were fixed in 10% neutral buffered formalin, paraffin embedded, sectioned at 4 micron and stained with Hematoxylin and Eosin (HE). Histologically, the heart showed a haemorrhagic laceration of the right

auricle associated with plurifocal petechial hemorrhagic infiltrating between the myocardial fibres (Fig. 3C). A peri-aortic hemorrhage without laceration of the vessel was also observed (Fig. 3D). Based on histological and macroscopic data, a definitive diagnosis of cardiac rupture was made. Finally, “hypovolemic shock”, due to right auricle and lung rupture, was considered as cause of death.

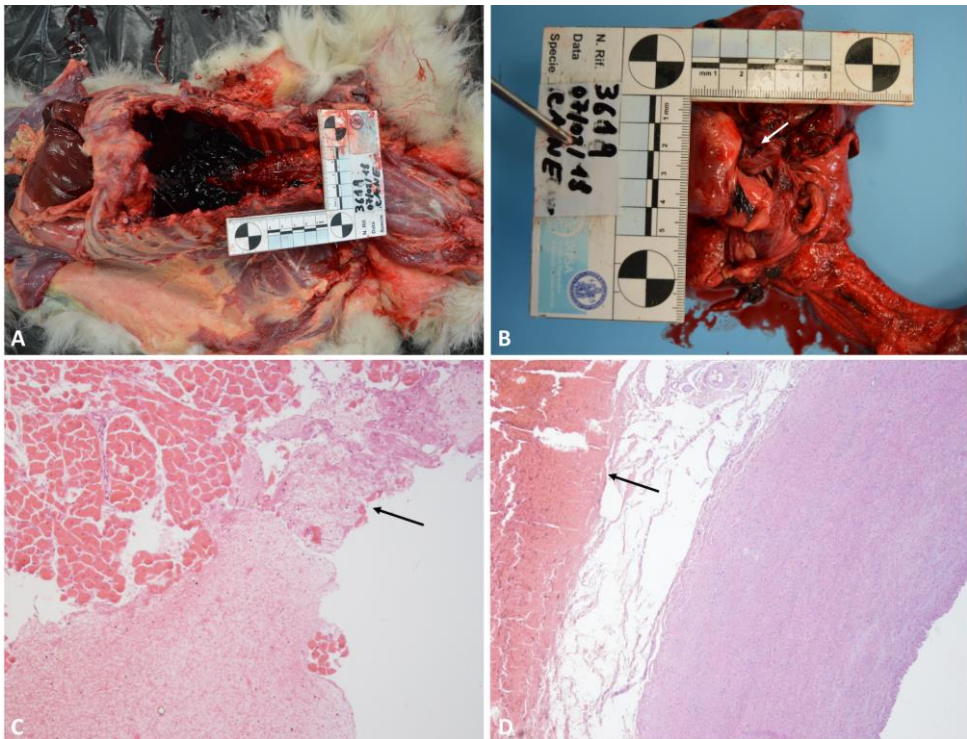


Figure 3: dog. Macroscopic examination: (A) clotted blood in the thoracic cavity (B) laceration of size 0.6 cm x 0.4 cm over the right auricle of the heart (arrow). Histopathological examination: (C) section from margin of tear; hemorrhagic laceration of the right auricle of the heart (arrow); H&E stain (original magnification 10x), (D) aorta: peri-aortic hemorrhage (arrow); H&E stain (original magnification 4x)

4.3. Discussion

Myocardial rupture is an injury frequently found at the autopsy following fatal accident due to blunt chest trauma in human (Modi et al., 2013; Brathwaite et al., 1990; Fulda et al., 1991). In contrast, in veterinary medicine, thoracic injuries commonly associated with non-penetrating trauma are: pneumothorax, pulmonary contusion, pulmonary laceration, hemothorax, ribs fractures, rhythm disturbances of the heart and cardiac contusion [Ressel et al., 2016; Snyder et al., 2001; Intarapanich et al., 2016]; In dog, cardiac laceration following NCT is only rarely reported in literature (Harpster et al. 1974). Furthermore, to the authors knowledge, this is the first report of cardiac laceration with NCT in a cat. Similarly, studies conducted on canine models of blunt chest trauma reported a low incidence of cardiac rupture following trauma. However, the same studies showed a high rate of mortality in dogs with cardiac laceration (Pandian et al. 1983; DeMuth et al. 1973). In general, though the mechanisms of myocardial injury are similar between man, cat and dog, differences exist in the nature of the initiating force. In human, the cause of hearth injury is often a driver impact with steering column during rapid deceleration in high-speed vehicular accident (Fulda et al., 1991; Wray, 2004); in contrast, in animals, the cause is a direct collision of the chest wall with a high-speed object (Harpster et al., 1974; Wray, 2004; Dinizlet al. 2007). As regard the mechanism of the injury, the transfer of the kinetic forces (Modi et al., 2007; Getz et al. 1986; Holanda et al., 2006), the compression of the heart between left and right thorax (Pandian et al. 1983; Wray, 2004) and the rapid increase of the intrathoracic pressure (Pandian et al. 1983; Wray, 2004) could be considered as causes of

lesion. Furthermore, in human medicine, an additional mechanism of injury is well described as a result of abdominal trauma (hydraulic ram effect (Chami et al. 2008)). A wide spectrum of potential injuries to the heart may result from NCT including: rhythm disturbances, myocardial contusion and myocardial rupture (Ressel et al. 2016; Pandian et al., 1983; DeMuth et al., 1973); the extent and the severity of the cardiac injury is determined by several biomechanical interactions including: 1) amount of force delivered to the body 2) the duration of the forces of acceleration 3) the total surface of the body on which the force is delivered 4) the plasticity of the tissues 5) relative movement between the contacting surface and the body of the animal 6) phase of the cardiac cycle at the time of injury [Ressel et al., 2016; Turan et al., 2010; Di Maio and Di Maio, 2011]. Force is an important factor in the extent of physical trauma and it is directly correlated with mass and acceleration according to Newtonian law of the dynamics:

Force = Mass x Acceleration

The moving and the energy that an object acquires following the application of a force is defined kinetic energy. This energy is the force transferred at another body when the chest trauma occurs and it is described as function of the object mass and velocity according to the formula of moving objects:

Kinetic energy = $\frac{1}{2}$ mass x velocity²

Considering this formula, it is possible to deduce that, as a result of a collision between the moving object and chest wall, the speed of the body, rather than its mass, determines the severity of the damage. Thus, injuries following trauma may present with different patterns of lesions, from contusion to laceration, as a result of different kinetic energy transferred by the moving object on the animal body.

In two of three presented cases, we observed cardiac rupture associated with ribs fractures and lung contusion and in only one case we did not observe any external chest injury. However, in all cases examined, the thoracic location of the injuries allowed to conclude that the cause of the cardiac rupture could be due to a direct impact of the chest wall with a high speed object with consequent transmission of the kinetic force and compression of the heart between left and right thorax. In a case, we observed complete cardiac rupture associated with tear of the pericardium; in this case, considering the laceration of the pericardium, a penetrating injury due to the ribs fractures could be considered as additional mechanism of injury. Finally, the difference in extension and severity of the cardiac rupture among cases examined could be attributed to the different extension and interaction of the variables described above; first of all the speed of the moving object.

4.4. Conclusion

Traumatic cardiac rupture is an uncommon diagnosis in veterinary medicine. However, the high mortality rate of cardiac laceration observed in canine models of blunt chest trauma make it an important challenge point for the veterinary forensic pathologist. During clinical examination, traumatic cardiac rupture could be an underdiagnosed injury, masked by the presence of other injuries or signs of shock. For these reasons, a proper macroscopic evaluation of the heart should always be carried out during forensic necropsies of polytrauma cases; a thorough understanding of the mechanisms underlying the heart rupture, associated with a complete forensic examination of the injuries, are important to define the manner of death and the causal relationship between trauma and death, especially during courts proceedings. Finally, the observation of a case of cardiac rupture without any external chest injuries highlight how, in clinical and forensic practice, the cardiac injury following blunt chest trauma should be ruled out even in the cases of absence of external chest injury

References

- Brathwaite CE, Rodriguez A, Turney SZ, Dunham CM, Cowley R, 1990. Blunt traumatic cardiac rupture. A 5-year experience, *Ann Surg*, 212 (6) 701–704.
- Chami MF, Nicholson W, Helmy T, 2008. Blunt cardiac trauma, *J Emerg Med*, 35 (2)127–133
- DeMuthJr WE, Lerner EH, Liedtke AJ, 1973. Non penetrating injury of the heart: an experimental model in dogs, *J. Trauma*, 13 (7)639–644.
- Di Maio VG, Di Maio D, *Forensic Pathology*, 2nd ed., CRC Press, Boca Raton, FL, 2001.
- Dinizl PPVP, Schwartzll DS, Collicchio-Zuanazel RC, 2007. Cardiac trauma confirmed by cardiac markers in dogs: two case reports, *Arq Bras Med. Vet. Zootec*, 59 (1)
- Fulda G, Brathwaite CE, Rodriguez A, Turney SZ, Dunham CM, Cowley RA, 1991. Blunt traumatic rupture of the heart and pericardium: a ten-year experience (1979–1989), *J Trauma*, 31 (2) 167–172.
- Getz BS, Davies E, Steinberg SM, Beaver BL, Koenig FA, 1986. Blunt cardiac trauma resulting in right atrial rupture, *JAMA*, 255 (6) 761–763.
- Harpster NK, VanZwieten MJ, Bernstein M, 1974. Traumatic papillary muscle rupture in a dog, *J. Am. Vet Med, Assoc.* 165 (12) 1074–1079.
- Holanda MS, Dominguez MJ, Lopez-Espadas F, Lopez M, Diaz-Reganon J, Rodriguez-Borregan JC, 2006. Cardiac contusion following blunt chest trauma, *Eur J Emerg Med*, 13 (6) 373–376,
- Intarapanich NP, McCobb EC, Reisman RW, Rozanski EA, Intarapanich PP, 2016. Characterization and comparison of injuries caused by accidental and non- accidental blunt force trauma in dogs and cats, *J Forensic Sci*, 61 (4)993–999

Modi K, Patel K, Chavali KH, Gupta SK, Agarwal SS, 2013. Cardiac laceration without any external chest injury in an otherwise healthy myocardium — a case series, *J Forensic Leg Med*, 20 (7) 852–854

Pandian NG, Skorton DJ, Doty DB, Kerber RE, 1983. Immediate diagnosis of acute myocardial contusion by two-dimensional echocardiography: studies in a canine model of blunt chest trauma, *J Am Coll Cardiol*, 2 (3) 488–496.

Piegari G, Iovane V, Carletti V, Fico R, Costagliola A, Biase DD, Prisco F, Paciello O, 2018. Assessment of Google Glass for Photographic documentation in veterinary forensic pathology, *JMIR mHealth and uHealth*, 6(9):e180

Ressel L, Hetzel U, Ricci E, 2016. Blunt force trauma in veterinary forensic pathology, *Vet Pathol*, 53 (5)941–961,

Snyder PS, Cooke KL, Murphy ST, Shaw NG, Lewis DD, Lanz OI, 2001. Electrocardiographic findings in dogs with motor vehicle-related trauma, *J Am Anim Hosp Assoc*, 37 (1) 55–63

Wray J, 2004. Blunt cardiac injury, *VRA*, 3 (1)3–10.

Turan AA,. Karayel FA, Akyildiz E, Pakis I, Uzun I, Gurpinar K, Atilmis U, Kir Z, 2010. Cardiac injuries caused by blunt trauma: an autopsy based assessment of the injury pattern, *J Forensic Sci*, 55 (1)82–84

Forensic Microbiology

Chapter 5

Contribution of forensic microbiology in sudden and unexpected death cases in young dog

5.1. Background

In human medicine the “sudden death” has been defined by the World Health Organization (WHO) as a rapid death during the first 24 hours after the onset of symptoms (Byard RW, 1994); in particular, the term “*sudden and unexpected infant death*” (SUID) is used to describe deaths that occur relatively suddenly and unexpectedly in children less than 1 year old (Kruger et al, 2018; Prtak et al, 2010; Bass et al, 2018). Infections are reported in literature as an important cause of SIUD following by metabolic or molecular disorders such as fatty acid oxidation, particularly mutations in the medium-chain acyl-coenzyme A dehydrogenase (MCAD) and genetic cardiac channelopathies (Kruger et al, 2018; Hannah and Bradley, 2009). The main pathogens reported in SUID cases are: *S. aureus*, *E. coli*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus pneumoniae*, *Group B Streptococcal (GBS)*, *respiratory syncytial virus (RSV)*, *cytomegalovirus (CMV)* and *Adenovirus* (Kruger et al, 2018, Rodríguez et al, 2006). However, a broad range of pathogens are reported in literature as cause or co-factor of death in SUID, such as: *Parvovirus B19*, *Epstein-Barr virus*, *Influenza A virus* and *mycobacterium tuberculosis* (Kruger et al, 2018; Fernandez-Rodriguez et al, 2006; Dempers et al. 2011; Dettmeyer et al. 2008; Zack et al. 2005 ; Dettmeyer et al. 2004). Furthermore, recent studies reported the relatively benign *Coxsackie Virus A16* as possible contributing factor in the sudden infant death in human (Astrub et al., 2016). For these reasons, the current SUID autopsy protocol in the UK (*Kennedy report*) and the international guidelines advocate a multi-agency approach to the investigation of all cases of SUID which

should be based not only on the findings of the post-mortem examination but also on a broad range of ancillary investigations, such as *bacteriological and virological analyses, metabolic analyses for fatty acid oxidation defects, histology, toxicology and biochemical assays in selected cases* (Kennedy, 2004; Bajanowski et al., 2007). However, among the cases of SUID, only 20% have a clear cause, while the most cases remain unexplained and categorized as *sudden infancy death syndrome (SIDS)* (Byard et al, 1994). Thus, SIDS is considered a sub-class of SUID where the cause of death remains unexplained even after forensic autopsy, ancillary tests, review of the circumstances of death and clinical history (Byard et al, 1994; Kruger et al. 2018). Although in human medicine a concept of sudden death has been well defined by the World Health Organization (WHO), in veterinary medicine a universal definition is missing. However, some authors defined sudden death in animals as death that accure in few minutes or severe hours to pre-existing disease or functional disorder (Parry, 2008). In veterinary medicine, previous studies reported a predominant infectious disease, affecting gastrointestinal system, as the main cause of death in puppies and young dogs (Eleni et al; Fleming et al, 2011). In contrast, neoplastic diseases appear to be the prevalent cause of death in adult dogs (Fleming et al, 2011). Among the infections, Canine Parvovirus Type 2 (CPV-2) is reported as one of the most common and important cause of morbidity and mortality in young dogs (17,18). Moreover, this virus is considered an important pathogen responsible of acute gastroenteritis and myocarditis in dog (Mylonakis et al, 2016; Nandi et al. 2010). Usually, the initial clinical signs of the infection are: *anorexia, depression, lethargy, and fever* following by *vomiting and diarrhea* that can range from mucoid to hemorrhagic

(Mylonakis et al, 2016; Nandi et al. 2010) However, as regard the sudden and unexpected death, despite the underlying causes have been investigated in dog sporadically (Olsen and Allen, 2000), to the best of our knowledge, no previous studies evaluated the microbiological findings in cases of sudden death in young dogs and, specifically, the frequency of infectious diseases in puppies and young dogs who died of sudden and unexpected death. In the light of these observations, the primary aim of this study is 1) to estimate the proportional mortality ratio (PMR) for “sudden and unexpected death” in puppies and young dogs 2) to determine the contribution of forensic microbiology in establishing a cause of death in sudden and unexpected death cases in puppies and young dogs.

5.2. Materials and methods

5.2.1. Study design and analytical validation of the results

An observational retrospective study of a total of 145 cases of young dead dogs consecutively presented by veterinary doctors, owners or law enforcements to the “Istituto Zooprofilattico del Mezzogiorno” (IZSM) of Portici city, Southern Italy, was carried out over a 3-years period (2015-2017). The submission forms were collected to abstain information about medical history and age of the animals. On the basis of the medical history, the animals were divided as follow: 1) dogs without clinical history of sudden and unexpected death (*expected deaths group*) and 2) dogs with clinical diagnosis of sudden and unexpected death (*sudden and unexpected deaths group*). According to WHO, sudden death was considered as non-

violent and unexpected death occurred less than 24 hours from the onset of symptoms. On the basis of age, the available data were categorized as follows: (Group 1) 5 days - 2 weeks; (Group 2) 2 weeks-1 month; (Group 3) 1-2 months; (Group 4) 2-3 months; (Group 5) 3-6 months; (Group 6) 6 -12 months. Each case included in the study was subjected to a complete forensic necropsy and bacteriological and virological analyses; Microbiological results and necropsy reports were both extracted from the IZSM informatic system (SIGLA).

Following the review of the necropsy reports, the cases of sudden death were included into two groups: **Group A**: deaths for suspect of viral or bacterial infection and **Group B**: death for clear non-infectious causes. For the purposes of this study, only the microbiological results from animals in the Group A were included in the study and the investigations were restricted to molecular tests (polymerase chain reaction or PCR for *Canine Parvovirus*, *Coronavirus*, *Adenovirus*, *Herpesvirus* and *Canine Distemper Virus*) for virological analysis and microbiological cultures for bacteriological examination.

Finally, Following the review of the necropsy reports, microbiological examinations and clinical histories of the sudden death cases, the final causes of death have been categorized as *explained or unexplained*. However, since determining the pathological significance of the microorganisms isolated during the necropsy is notoriously difficult as highlighted frequently in the literature (Morris et al., 2006; Wilson et al, 1993; Ridgway and Harvey, 2009), for the purposes of this study, viruses detected by PCR were considered to be cause of death, only whether associated by typical macroscopic changes observed during the

anatomopathological examination. In addition, during the interpretation of the bacteriological and virological analyses, the location from which the micro-organisms were isolated, their pathogenic potential, the correlation with injuries observed during the necropsy, the multisite location of the agents, the age of the dog and the composition of the normal flora were taken into consideration.

5.2.2. Statistical analysis

Frequency of *sudden and unexpected deaths*, *expected deaths* and the *total deaths (sudden deaths + expected deaths)* were evaluated and stratified by age classes. Furthermore, we estimated the proportional mortality ratio (PMR) for “*sudden and unexpected death*” in each assessed age group. Chi-square test was used to assess differences in distribution between expected deaths and sudden unexpected deaths among age groups.

5.3. Results

Out of the 145 examined reports, we found 21 cases of sudden and unexpected death and 124 cases of expected death during the 3-years study period. The proportional mortality ratio of sudden death was therefore 14.48 % compared with 85.52% for expected death. Furthermore, chi-squared test showed a significant difference in frequencies between expected death and sudden death among assessed age groups ($p < .05$). The highest frequency of expected death was observed in animals of the group 2 (100% of the cases), 3 (87,7% vs 12,5 %), 4 (97,2% vs 12,8%) and 5 (93,3 vs 6,7%). In contrast, the highest frequency of sudden death was found in the animals of the group 1 (58,8% vs 41,2) and 6 (37,5% vs 62,5%). Tab. 1 summarized the frequency and percentage of sudden and unexpected deaths, expected deaths and the frequency of total deaths (sudden deaths + expected deaths) stratified by age classes.

Tab. 1 frequency and percentage of sudden and unexpected deaths, expected deaths and the frequency of total deaths stratified by age groups

Age Group	Sudden death	Expected death	Total deaths
Group 1	10 (58,8%)	7 (41,2%)	17
Group 2	0	14 (100%)	14
Group 3	5 (12,5%)	35 (87,5%)	40
Group 4	1 (2,8%)	35 (97,2%)	36
Group 5	2 (6,7%)	28 (93,3%)	30
Group 6	3 (37,5%)	5 (62,5%)	8
Total	21	124	145

Overall, of the 21 sudden unexpected death cases, 10/21 dogs presented less than 15 days of age (group 1) , 0/21 an age range between 15 days and 1 month (group 2), 5/21 an age between 1-2 months (group 3), 1/21 presented 2-3 months of age (group 4), 2/21 an age between 3-6 months (group 5) and 3 /21 an age range between 6 months and one year (group 6). Following the review of the necropsy reports, the cause of death was classified as presumed viral or bacterial infections in 16 cases and due to causes other than infection in the remaining 5 cases. As regard the microbiological examinations, virological investigation was performed with a panel of viruses tested by PCR (Parvovirus, Coronavirus, Adenovirus, Herpesvirus and Canine Distemper Virus) and the bacteriological examination was performed with microbiological culture. Among cases in which the infection was suspected during necropsy, positive microbiological results were observed in all assessed cases (16/16) (Tab.2).

Tab. 2 Viruses or bacteria detected in sudden and unexpected death cases

Pathogen	N° of cases
Canine Parvovirus	13
Clostridium perfringens typa A	6
E. Coli	6
Adenovirus	3
Canine Distemper Virus	2
Streptococcus dysgalactiae	1
Streptococcus sanguinis	1
Rotavirus	1
Pasteurella spp.	1

However, for the purposes of this study, the microbiological findings were interpreted considering a broad range of variables, such as location from which a micro-organism was isolated, its pathogenic potential, the correlation with macroscopic injuries, the multisite location of the agents, the age of the dog and the composition of the normal flora. Therefore, after review of necropsy and microbiological reports, pathogens were considered cause of death in only 12 out of 16 cases. In particular, among assessed cases, the main cause of death was a viral infection due to *Canine Parvovirus type 2* (8/16) following by bacterial gastroenteritis due to *Clostridium perfringens type A* and *E. Coli* (1/16), pneumonia due to co-infection *Canine Distemper Virus* and *Pasteurella spp.* (1/16) and viral and bacterial infection due to co-infection *Canine parvovirus type 2* and *E.coli* (2/16). Finally, in 4 out of 16 cases, microbiological results did not allow to explain the injuries observed during the necropsy. Therefore, the causative agent of infection was considered undetermined after microbiological examination. Table 3 summarized the clinical backgrounds, pathological findings, microbiological results and the causes of death of the sudden death cases.

Tab 3. Microbiological and anatomopathological findings of the studied dogs

Group	Clinical background	Pathological findings	Virological examination	Bacteriological examination	Cause of death
1	Acute respiratory insufficiency	visceral congestion, pulmonary edema, hemorrhagic gastroenteritis	Canine parvovirus type 2a (lung, liver, brain and intestine)	Streptococcus dysgalactiae (isolated from the lung tissue samples)	viral infection
1	Acute respiratory insufficiency	visceral congestion, pulmonary edema, severe broncho-pneumonia , enteritis	Canine parvovirus type 2a (spleen)	Streptococcus sanguinis (isolated from the intestine tissue samples)	Undetermined: severe pneumonia due to unexplained causes
1	Acute respiratory insufficiency	visceral congestion, multifocal pulmonary hemorrhages, hemorrhagic enteritis	Canine parvovirus type 2b and 2c (lung, liver, spleen and intestine)	Clostridium perfringens, Detection of Clostridium perfringens alpha toxin (isolated from the intestine tissue samples)	Viral infection
1	Acute respiratory insufficiency	lobar pneumonia, catarrhal enteritis	Canine parvovirus type 2b, (intestine) <i>Canine distemper virus (lung)</i>	Pasteurella spp. (isolated from the lung tissue samples)	viral and bacterial pneumonia
1	Acute respiratory insufficiency	visceral congestion, multifocal pulmonary hemorrhages, hemorrhagic enteritis	Canine parvovirus type 2b and 2c (lung, liver, brain and intestine)	E.coli, Clostridium perfringens, Detection of Clostridium perfringens alpha toxin (isolated from the intestine tissue samples)	Viral infection
1	Acute respiratory insufficiency	visceral congestion, pulmonary edema, Focal broncho-pneumonia, hemorrhagic gastroenteritis	Canine parvovirus type 2a (lung and intestine)	No bacteria detected	Viral infection
1	hypovolemic shock	visceral congestion, hemorrhagic enteritis	Canine parvovirus type 2a (lung, spleen and intestine)	No bacteria detected	Viral infection
3	sialorrhea, unilateral eye swelling, muscle stiffness, a single episode of vomiting	visceral congestion, pulmonary edema, hemorrhagic enteritis	No virus detected	E.coli, Clostridium perfringens, Detection of Clostridium perfringens alpha toxin (isolated from the intestine and lung tissue samples)	Bacterial gastroenteritis
3	neurological symptoms	visceral congestion, bi-lateral pneumonia, pulmonary edema segmental catarrhal enteritis	Rotavirus (detected in the intestine)	E.coli (isolated from the intestine tissue samples)	Undetermined: severe pneumonia due to unexplained causes
3	acute gastrointestinal symptoms	visceral congestion, hemorrhagic enteritis, focal pneumonia	Canine parvovirus type 2a, (lung, liver and intestine) <i>Adenovirus (intestine)</i>	Clostridium perfringens, Detection of Clostridium perfringens alpha toxin (isolated	Viral gastroenteritis

Forensic microbiology in sudden death cases

				from the intestine tissue samples)	
3	Vomiting	visceral congestion, pulmonary congestion, enteritis, abdominal, thoracic and pericardial effusion, multifocal pulmonary hemorrhage	No virus detected	E.coli (isolated from intestine tissue samples)	Undetermined: insufficient findings to explain death
5	Acute respiratory insufficiency	pulmonary congestion, segmental catarrhal enteritis	Canine parvovirus type 2a (lung, liver, intestine)	E.coli (isolated from lung, liver, spleen and intestine tissue samples)	Viral and bacterial gastroenteritis
5	a single episode of diarrhea	pulmonary congestion, pulmonary edema, segmental hemorrhagic enteritis	Canine parvovirus type 2a, (lung, liver, intestine and spleen) <i>Adenovirus (lung)</i>	No bacteria detected	Viral gastroenteritis
6	Acute respiratory insufficiency	thoracic effusion, visceral congestion, multifocal hemorrhage, severe hemorrhagic enteritis	Canine parvovirus type 2a and 2c, (lung, liver, intestine and spleen) Canine distemper virus (lung) Adenovirus (lung)	No bacteria detected	Viral gastroenteritis
6	lack of appetite and fever for 12h	multifocal hepatic necrosis, hemorrhagic enteritis	Canine parvovirus type 2b (lung, liver, brain and intestine)	E. coli. Clostridium perfringens, Detection of Clostridium perfringens alpha toxin (isolated from the lung, liver and intestine tissue samples)	Viral and bacterial gastroenteritis
6	single episode of diarrhea	Congestion of the spleen, abdominal effusion	Canine parvovirus type 2a (lung)	Clostridium perfringens, Detection of Clostridium perfringens alpha toxin (isolated from intestine tissue samples)	Undetermined: insufficient findings to explain death

5.4. Discussion

In human forensic pathology, the autopsy guidelines for the cases of SUDI are primarily based on the “Kennedy Report” (Kennedy et al. 2004). This protocol and the published international guidelines advocate a multidisciplinary approach to the investigations of all cases of SUID, which should be based not only on the findings of the post-mortem macroscopic examination, but also on a broad range of ancillary investigations, such as bacteriological and virological analyses, radiology, histological examinations, toxicology and biochemical assays in selected cases. Although a broad range of tests have been proposed in cases of SUID in human forensic medicine, in the present study, we focused on the contribution of post-mortem microbiology in establishing a cause of death in the sudden death cases in young dog. The results of this study showed a low frequency of sudden death in young dog with a percentage of 14,48 % of the total observed deaths. Furthermore, chi-squared test showed a significant difference in frequencies between *expected and sudden deaths* among assessed age groups ($p < .05$). In particular, the highest frequency of sudden death was observed in dogs with age less than 15 days (47% of the total cases of sudden death and 58,8% of the total deaths observed in the Group 1). In contrast, the highest frequency of expected death was observed in animals of the group 2 (100% of the cases), 3 (87,7% vs 12,5 %), 4 (97,2% vs 12,8%) and 5 (93,3 vs 6,7%). This difference could be due to the immaturity of the immune system of puppies under 6/12 weeks of age (Day, 2007). Indeed, the endotheliochorial placentation of this specie is relatively impenetrable to the transfer of maternal immunoglobulin (Day, 2007). Thus, the immune protection of the puppies during the first weeks of life depends

on the ingestion of the maternal colostrum antibodies (MCA) (Day, 2007). In the absence of passive transfer of MCA, the newborn puppies are able to develop an immune response to antigens only at 2/3 weeks of age. Therefore, any delay in colostrum intake or reduction of colostrum ingestion leads to reduction of the immune protection of the animals (Day, 2007; Decaro and Buonavoglia, 2012; Mila et al, 2014). In these conditions, viruses or bacteria could replicate and spread quickly, leading to death of the puppies without development of characteristic symptoms. Furthermore, intrauterine malnutrition associated with excessive multiple pregnancy, maternal malnutrition, congenital defects, dystocia or prolonged labour and the associated hypoxia or anoxia could be considered additional causes of sudden death in this range of life. In particular, in our study, the review of necropsy reports allowed us to identify 5 cases of sudden death due to clear non-infectious causes and 16 cases of death due to presumed viral or bacterial infections. Among cases in which the infection was suspected during the necropsy, positive microbiological results were observed in all assessed cases (16/16). However, as frequently highlighted in the human literature, the detection of pathogens in sudden death cases does not necessarily imply a correlation between pathogens and death. In particular, the link between infection and death must be evidenced by the presence of severe and specific injuries during anatomopathological examination or, if available, histological examination. Therefore, after review of the necropsy findings, detected pathogens were considered main cause of death in only 12 out of 16 cases, while, in the remaining 4 cases, the microbiological results did not allow to explain the injuries observed during the necropsy. Therefore, the causative agent of death was considered undetermined after

microbiological examination. The negative findings observed in our study could suggest 1) a non-infectious cause of death of the assessed animals or 2) a death due to viruses not detected by the virological panel in use in this study. Indeed, there are a wide range of viruses potentially pathogens in young dogs, including both DNA and RNA viruses. However, our virological panel was limited to the detection of 5 specific viruses: *Canine Parvovirus*, *Coronavirus*, *Adenovirus*, *Herpesvirus* and *Canine Distemper Virus*. As regard the positive results, viral infection due to *Canine Parvovirus type 2* (CPV-2) was the most common cause of death observed in our study. CPV-2 is considered a causative agent of acute gastroenteritis and myocarditis (Mylonakis et al., 2016; Nandi et al, 2010). Furthermore, it is reported in literature as one of the most common and important cause of morbidity and mortality in young dogs (Mylonakis et al., 2016;Nandi et al, 2010). Usually, the initial clinical signs of the infection are: *anorexia, depression, lethargy, and fever following by vomiting and diarrhea that can range from mucoid to hemorrhagic* (Mylonakis et al., 2016;Nandi et al, 2010). However, it is also reported as important cause of sudden death in puppies (Mylonakis et al., 2016, Hayes et al, 1979, Stann et al, 1984). As regard the bacteriological examination, the most common isolated bacteria were *Clostridium perfringens type A* (6/19) and *E.coli* (6/19). However, they were considered cause of death in only one case. Indeed, *Clostridium Perfringens* and *E.coli* are considered a normal component of the intestinal flora (Goldstein et al, 2012; Bettelheim and Goldwater, 2015). Similarly, the alpha toxin gene of *Clostridium perfringens* may be found in asymptomatic dogs as part of the normal intestinal microflora. However, in some cases, *E.coli* can be cause of pleuro-pneumonitis (Handt et al., 2003),

gastroenteritis (Astrid et al. 2016), urogenital infections, cholangitis, cholangiohepatitis (Beutin, 1999) and septicemia (Beutin, 1999) in both human and animals. Similarly, *Clostridium Perfringens* type A has been associated with a wide range of enteric diseases, such as abomasitis and enteritis in ruminants, necrotic enteritis in broiler chickens and mild or fatal acute hemorrhagic enteritis in dog. Furthermore, this bacteria has been sporadically reported in literature as a cause of sudden and unexpected death in dog (Schlegel et al, 2012). Unfortunately, no gold standard tests for the diagnosis of enteritis associated with *C. perfringens* are described in literature (Marks and Kather, 2006). Thus, generally, the clinical signs, anatomopathological findings, molecular diagnostic evidence and the absence of other pathogens must be examined before making a diagnosis. In our case, the absence of other viruses or bacteria, associated with specific anatomopathological findings of hemorrhagic enteritis, supported the diagnosis of enteritis due to *C. perfringens* and *E.coli* as finally cause of death. Finally, this study allowed to detect a wide range of pathogens that, after review of the necropsy and microbiological reports, were not considered main cause of death of the animals, such as *Adenovirus*, *Rotavirus*, *Streptococcus sanguinis*, *Dysgalactie* and, in some cases, *E.coli* and *Clostridium perfringens*. Therefore, further studies will be needed to evaluate the possible contributing factor of these pathogens in the cases of sudden and unexpected death in young dog.

5.5. Conclusion

Sudden death is an uncommon diagnosis in veterinary medicine. However, the high frequency of sudden death observed in dogs less than 15 days of age makes it an important challenge point for the veterinary forensic pathologist. The high frequency of viruses and bacteria detected in our study confirms the relevance of performing complete bacteriological and virological investigations in all cases of sudden death in young dog. The results of this study suggest that our panel of PCR assays, associated with bacteriological analysis will permit a rapid detection and type-specific identification of pathogens cause of death in most cases of sudden death in young dogs. Finally, these results will provide a valuable epidemiological tool for investigation of the sudden death in young dog.

- Astrid B. Kjaergaard, Anthony P. Carr, M. Casey, 2016. Gaunt Enteropathogenic Escherichia coli (EPEC) infection in association with acute gastroenteritis in 7 dogs from Saskatchewan Can Vet J, Sep; 57(9): 964–968.
- Astrup BS, Johnsen IBG, Engsbro AL, 2016. The role of Coxsackievirus A16 in a case of sudden unexplained death in an infant – A SUDI case, Forensic Sci Int, 259 e9–e13.
- Bass JL, Gartley T, Lyczkowski DA, Kleinman R, 2018. Trends in the Incidence of Sudden Unexpected Infant Death in the Newborn: 1995-2014. J Pediatr, May;196:104-108.
- Byard RW, 1994. Sudden death – an overview, in: R.W. Byard, S.D. Cohle (Eds.), Sudden Death in Infancy, Childhood, and Adolescence, Cambridge University Press, pp. 1–4.
- Bajanowski T, Vege A, Byard RW, Krous HF, Arnestad M, Bachs L et al., 2007. Sudden infant death syndrome (SIDS) – standardised investigations and classification: recommendations. Forensic Sci Int, 165:129–143
- Beutin L, 1999. Escherichia coli as a pathogen in dogs and cats. Vet Res, 30(2-3):285-98.
- Bettelheim KA, Goldwater PN, 2015. Escherichia coli and Sudden Infant Death Syndrome. Front Immunol, 6: 343.
- Day M J, 2007. Immune System Development in the Dog and Cat. J. Comp. Path, Vol.13
- Decaro N, Buonavoglia C, 2012. Canine parvovirus—A review of epidemiological and diagnostic aspects, with emphasis on type 2c. Vet Microbiol Feb 24;155(1):1-12.
- Dempers J, Ann SM, Wade SA, Kinney HC, Odendaal HJ, Wright CA, 2011 Progressive Primary Pulmonary Tuberculosis Presenting as the Sudden Unexpected Death in Infancy: A Case Report. Forensic Sci Int, 2011 Mar 20; 206(1-3): e27–e30. doi: 10.1016/j.forsciint.2010.07.018

Dettmeyer R, J.P. Sperhake, J. Müller, B. Madea, 2008. Cytomegalovirus-induced pneumonia and myocarditis in three cases of suspected sudden infant death syndrome (SIDS): Diagnosis by immunohistochemical techniques and molecularpathologic methods, *Forensic Sci. Int.* 229–233.

Dettmeyer R, Baasner A, Schlamann M, Padosch SA, Haag C, Kandolf R, Madea B, 2004. Role of virus-induced myocardial affections in sudden infant death syndrome: a prospective postmortem study. *Pediatr Res*, 2004 Jun;55(6):947-52.

Eleni C, Panetta V, Scholl F., Scaramozzino P. Causes of death in dogs in the province of Rome (Italy). *Vet Ital.* 2014 Apr-Jun;50(2):137-43.

Fernández-Rodríguez A, Ballesteros S, de Ory F, Echevarría JE, Alvarez-Lafuente R, Vallejo G, Gómez J, 2006. Virological analysis in the diagnosis of sudden children death: a medico-legal approach. *Forensic Sci Int*, Aug 10;161(1):8-14.

Fleming JM, Creevy KE, and Promislow DEL, 2011. Mortality in North American Dogs from 1984 to 2004: An Investigation into Age-, Size-, and Breed-Related Causes of Death. *J Vet Intern Med*, 25:187–198

Goldstein MR, Kruth SA, Bersenas AME, Holowaychuk MK, Weese JS, 2012. Detection and characterization of *Clostridium perfringens* in the feces of healthy and diarrheic dogs. *Can J Vet Res*. 2012 Jul; 76(3): 161–165.

Hannah CK, Bradley TT, 2009. The Sudden Infant Death Syndrome. *N Engl J Med*, Aug 20; 361(8): 795–805.

Handt LK, Stoffregen DA, Prescott JS, Pouch WJ, Ngai DT, Anderson CA, Gatto NT, DebRoy C, Fairbrother JM, Motzel SL, Klein HJ, 2003. Clinical and microbiologic characterization of hemorrhagic pneumonia due to extraintestinal pathogenic *Escherichia coli* in four young dogs. *Comp Med*, Dec;53(6):663-70.

- Hayes MA, Russell RG, Babiuk LA, 1979 Sudden death in young dogs with myocarditis caused by parvovirus. *J Am Vet Med Assoc*, Jun 1;174(11):1197-203.
- Kennedy H, 2004. Sudden unexpected death in infancy. A multi-agency protocol for care and investigation. The report of a working group convened by The Royal College of Pathologists and The Royal College of Paediatrics and Child Health. London: The Royal College of Pathologists/The Royal College of Paediatrics and Child Health
- Kruger MM, Martin LJ, Maistry S, Heathfield LJ, 2018. A systematic review exploring the relationship between infection and sudden unexpected death between 2000 and 2016: A forensic perspective. *Forensic Sci Int*, Aug;289:108-119.
- Marks SL and Kather EJ, 2006. Clostridium perfringens- and Clostridium difficile-associated diarrhea. In: Greene CE, editor. *Infectious Diseases of the Dog and Cat*. 3rd ed. St Louis, Missouri: Saunders Elsevier,. pp. 363–366.
- Mila H, Grellet A, Desario C, Feugier A, Decaro N, Buonavoglia C, 2014. Chastant-Maillard S. Protection against canine parvovirus type 2 infection in puppies by colostrum-derived antibodies. *J Nutr Sci*, 3: e54.
- Morris JA, Harrison LM, Partridge SM, 2006. Postmortem bacteriology: a re-evaluation. *J Clin Pathol*, 59:1–9.
- Mylonakis ME, Kalli I, Rallis TS, 2016. Canine parvoviral enteritis: an update on the clinical diagnosis, treatment, and prevention. *Vet Med (Auckl)*, 7: 91–100
- Nandi S, Kumar M, 2010. Canine Parvovirus: Current Perspective. *Indian J Virol*, Jun; 21(1): 31–44.
- Olsen TF, Allen AL, 2000. Causes of sudden and unexpected death in dogs: a 10-year retrospective study. *Can Vet J*, Nov; 41(11): 873–875.
- Parry NM, 2008. Sudden and unexpected death in small animal patients : Part 1. *Companion Animal*, v. 13, n. 8, p. 1-6

- Prtak L, Al-Adnani M, Fenton P, Kudesia G, Cohen MC, 2010. Contribution of bacteriology and virology in sudden unexpected death in infancy. *Arch Dis Child*, 2010 May;95(5):371-6.
- Ridgway EJ, Harvey D, 2009. The hospital autopsy: a manual of fundamental autopsy practice. In: Burton J, Ruttly G, eds. *The hospital autopsy*, 3rd edn. London: Hodder Education
- Sasaki J, Goryo M, Asahina M, Makara M, Shishido S, Okada K, 1999. Hemorrhagic enteritis associated with *Clostridium perfringens* type A in a dog. *J Vet Med Sci*, Feb;61(2):175-7.
- Schlegel JB, Dreumel TD, Slavić D, Prescott JF, 2012. *Clostridium perfringens* type A fatal acute hemorrhagic gastroenteritis in a dog. *Can Vet J*, May; 53(5): 555–557.
- Shapiro-Mendoza CK, Camperlengo L, Ludvigsen R, Cottengim C, Anderson RN, Andrew T, Covington T, Hauck FR, Kemp J, MacDorman M, 2014. Classification System for the Sudden Unexpected Infant Death Case Registry and its Application. *Pediatrics*, Jul; 134(1): e210–e219.
- Stann SE, DiGiacomo RF, Giddens WE, Evermann JF, 1984. Clinical and pathological features of parvoviral diarrhoea in dogs. *J Am Vet Med Assoc*, 185:651–654
- Weese JS, Greenwood SJ, Staempfli HR, 2001. Recurrent diarrhea associated with enterotoxigenic *Clostridium perfringens* in 2 dogs. *Can Vet J*, Apr; 42(4): 292–294.
- Wilson SJ, Wilson ML, Reller LB, 1993. Diagnostic utility of postmortem blood cultures. *Arch Pathol Lab Med*, 117:986–8.
- Zack F, Klingel K, Kandolf R, Wegener R, 2005. sudden cardiac death in a 5-year-old girl associated with parvovirus B19 infection. *Forensic Sci Int*, Dec 1;155(1):13-7.