Antonio Pugliese · Alberto Gaiti
Cristiano Boiti *Editors*

Veterinary Science

Current Aspects in Biology, Animal Pathology, Clinic, and Food Hygiene

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Part I
Biology and Reproduction

Chapter 1
Nucleofection of Ovine Amniotic Fluid-Derived Mesenchymal Stem Cells

V. Curini, A. Colosimo, A. Mauro, M. Turriani, A. Gloria, M. Mattioli, and B. Barboni

Abstract Amniotic fluid has attracted increasing attention in recent years as a possible source of stem cells. Amniotic stem cells have high differentiation ability and low immunogenicity, and are thus an ideal candidate for stem cell-based regenerative therapy. To assess their potential applicability, preclinical studies have been initiated. In this context, the availability of GFP-expressing cells could be extremely useful as a protein marker to visualize transferred stem cells within damaged tissue. In the present study, nucleofection, a recent electroporation-based technique, was used to transfect GFP-expressing plasmids into ovine amniotic fluid-derived stem cells. The study shows that this transfection method can be used to generate stable transgene expression in amniotic stem cells without altering their differentiation potential.

Keywords Amniotic cells • Nucleofection • Ovine • Staminality markers

1.1 Introduction

In the field of regenerative medicine, stem cells represent a useful tool for restoring the function of damaged tissues and organs. Recently, scientists have given special attention to amniotic fluid-derived cells because these cells have a high degree of plasticity, as they are able to differentiate in vitro into neurons, osteoblasts, fibroblasts, adipocytes, chondrocytes, hepatocytes, and endothelial cells (Parolini et al. 2009). Immunocytochemistry and gene expression analyses confirmed the presence of specific markers, such as OCT-4A, SOX2, NANOG, and TERT, as

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indispensable for maintaining a cell in an undifferentiated state. Other important biological characteristics are low immunogenicity and immunoregulatory features that have been observed in vitro and in vivo that together allow for the use of these cells for allo/xenografts. Amniotic cells also have anti-inflammatory, antimicrobial, and antifibroblastic features, and do not show any evidence of tumor formation. Unlike embryonic stem cells, amniotic cells do not present ethical problems for their recovery from humans (Insausti et al. 2010). For these reasons, particular attention has been directed to stem cells derived from amniotic fluid and membranes as an alternative source of mesenchymal stem cells (MSC) that are useful in the field of regenerative therapy.

In recent studies, amniotic cells have been used in preliminary tests with animal models to test their ability to regenerate damaged tissue following injuries of the neuronal system (i.e., Parkinson's disease), kidney, bone marrow, or myocardium. However, preclinical studies are still necessary to demonstrate the regenerative capacity and to ensure the long-term safety of the treatment.

In this context, it is relevant that the animal model has morphofunctional characteristics similar to humans. For this reason, sheep are considered an optimal model for studying bone, skeletal muscle, and tendon diseases. To assess the regenerative effect of transferred stem cells into a pathologically or experimentally damaged tissue, it is necessary to have cells that express a marker, such as green fluorescent protein (GFP), which allows for their identification following transfection. There are different methods for transferring foreign DNA into a cell. Viral methods that use modified virus (adenovirus, lentivirus, or retroviruses) are the most efficient techniques and are also technically demanding because they require specific safety conditions. In contrast, nonviral-based methods, including chemical systems, such as lipofection, and physical systems, such as electroporation, are less efficient than viral methods, especially since they trigger higher levels of cell mortality.

Recently, a new nonviral method based on electroporation, called nucleofection, has been described. This technique consists of a combination of cell-specific solutions and optimized electrical parameters that lead to increased efficiency of gene transfer to the cell nucleus (Zaragosi et al. 2007). In addition, this method is effective on many primary cell lines that are typically difficult to transfect by nonviral methods.

The aim of the present study was to optimize a nucleofection program that was already tested on human MSC for ovine stem cells isolated from amniotic fluids (AFSCs). Optimization was evaluated by examining cell viability, efficiency of gene transfer in the short and long term, maintenance of stemness characteristics, and osteo-plasticity in vitro.

1.2 Materials and Methods

1.2.1 Isolation and Culture of AFSCs

AFSCs were isolated from amniotic fluid samples by centrifugation and resuspended in α-MEM growth medium supplemented with 20% FCS, 1% penicillin/streptomycin, 1% L-glutamine, and 5 ng/ml fibroblast growth factor (FGF). Cells were incubated in a Petri dish in a humidified 38.5°C/5% CO_2 incubator. At 80% confluency, cells were lifted with 0.05% trypsin EDTA and counted using a Burker chamber.

1.2.2 Nucleofection

Cells (6×10^5) were resuspended in 100 μl of Human MSC Nucleofector Solution (Amaxa Biosciences) with 2.5 μg pAcGFP-N1 vector (Clontech) and nucleofected with the U-23 or C-17 programs of a Nucleofector II device. These programs are specific for human MSCs. Immediately after nucleofection, cells were plated into 90-mm dishes and incubated for 48 h. After the incubation, gentamycin (400 μg/ml) was added to growth medium to select for the cells with internalized and integrated plasmid. The selection was performed for 2 weeks.

1.2.3 GFP Expression Assessment

Samples of AFSCs nucleofected with either of the two programs were used to assess fluorescence as determined by GFP expression. Cells were stained with propidium iodide (PI), resuspended in 500-μl of phosphate-buffered solution (PBS), and analyzed on a flow cytometer Coulter Epics XL (Beckman Coulter). The fluorescence signals of GFP and PI were measured in channels FL1 and FL2, respectively, with a logarithmic scale.

1.2.4 Assessment of Stemness Markers

Immunocytochemistry and RT-PCR were used to assess stemness of nucleofected cells. Cells (2×10^4) were fixed in 4% paraformaldehyde/PBS for 15 min at room temperature and were analyzed by immunocytochemistry for the expression of SOX2, NANOG, and TERT pluripotency markers. For RT-PCR analysis, total RNA was extracted from 1×10^6 AFSCs using TRI Reagent (Sigma). Reverse

transcriptase reactions and gene-specific PCRs were performed to evaluate mRNA expression of *OCT4A*, *SOX2*, and *NANOG*.

1.2.5 Clonal Selection

The remaining cells were harvested by trypsinization and resuspended in growth medium supplemented with antibiotics. Suspended cells (200 μl for a total of 4×10^3 cells) were used for serial dilutions in 96-well flat-bottom plates. At confluency, the wells with the highest percentage of fluorescent cells were evaluated by fluorescent microscopy. Selected clones were plated in 12-well plates, and successively into 90-mm dishes. GFP expression was analyzed on a flow cytometer.

1.2.6 In Vitro Osteogenic Differentiation

AFSCs were induced to osteo-differentiate with differentiation medium (αMEM supplemented with 10% FCS, 1 mM ascorbic acid, 1 M β-glycerol phosphate, 500 μM dexamethasone, 1% of penicillin/streptomycin, and 1% of -glutamine). Cells were incubated for 7 days in a humidified 38.5°C/5% CO_2 incubator. Mineralization was evaluated by Alizarin Red S staining.

1.3 Results

The viability of nucleofected AFSCs, calculated as the ratio of the number of adhered cells to the total number of cells exposed to nucleofection, was 1.6 and 12% for cells nucleofected with the U-23 and C-17 programs, respectively. The efficiency of gene transfer, measured as the percentage of GFP-positive cells by flow cytometry, was found to be 28.5% and 37.2% for the U-23 and C-17 programs, respectively.

To test if the nucleofection influenced the characteristics of amniotic stem cells, we evaluated the expression of molecular markers specific to stem cells and the ability of these cells to osteo-differentiate in vitro. Immunocytochemistry analysis, performed before and after nucleofection, showed the presence of stemness markers (SOX2, NANOG, and TERT) in both nucleofected and control cells. The localization of the markers was nuclear in 40% of the AFSCs. Gene expression analysis of stemness markers using RT-PCR showed only the presence of *NANOG* mRNA, while *SOX2* and *OCT4A* were not expressed in nucleofected cells or in the control. Osteogenic differentiation in vitro was determined by histological staining (Alizarin Red) that indicated deposits of hydroxyapatite. Histological analysis

showed after only 7 days of differentiation in vitro the presence of Alizarin Red-positive crystals in GFP-positive cells and in nonnucleoporated AFSCs, while cells maintained in growth medium continued to proliferate without increased calcium deposits.

Nucleofected cells were proliferated in vitro in selective medium to assess the stability of the transfection over time and to select for individual clones. After serial dilutions were made, a single clone containing a high percentage of fluorescent cells was selected using fluorescent microscopy and flow cytometry for each nucleofection program. The clone obtained using the U-23 program had a 95.49% rate of fluorescence with an intensity of 164, and the clone obtained using the C-17 program had a 93% rate of fluorescence with an intensity of 112.

1.4 Discussion

Nucleofection was a very efficient method for a primary cell line. The two nucleofection programs optimized for human MSCs were also suitable for ovine amniotic stem cells. The C-17 program was less efficient at transfecting the cells compared to the U-23 program; however, C-17 resulted in a lower percentage of cell mortality, the highest percentage viability, and the highest percentage of transfected ovine AFSCs. Furthermore, by serial dilution in 96-well plates, single colonies were isolated that were more morphologically homogeneous than the original population, and nearly all cells expressed GFP.

Nucleofected AFSCs have been cultured for more than a month confirming that nucleofection is a transfection method that is useful for long-term expression because the exogenous plasmid is integrated into the genomic DNA of the host cell and continues to be replicated during cell proliferation. Immunocytochemistry and RT-PCR analyses indicated that nucleofected cells were unchanged based on their expression of stemness markers and their ability to osteo-differentiate.

The availability of stable single clones of pluripotent stem cells expressing the *GFP* reporter gene opens the possibility of developing preclinical models to both demonstrate the regenerative capacity of amniotic stem cells and investigate the mechanisms underlying their integration in experimentally injured tissues.

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Chapter 2
Osteogenic Potential of Sheep Amniotic Epithelial Stem Cells

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Abstract Amniotic membranes and amniotic fluid have attracted increased attention as possible sources of stem cells to be used for clinical application in regenerative medicine. However, the biology of these cells and the signals required to effectively direct their differentiation are still largely unknown. This work was designed to define stemness properties in sheep amniotic epithelial cells (AECs) and evaluate their osteogenic potential in vitro. Sheep AECs express cell adhesion markers CD29 and CD49 and the pluripotent stem cell markers Oct4, SOX2, Nanog, and TERT that are typical of mesenchymal stem cells. Under specific inductive culture conditions, AECs undergo prompt osteogenic differentiation with extensive extracellular matrix mineralization, and the remarkable plasticity of these cells is progressively reduced after six expansion passages.

Keywords Calcein • Cell culture • Differentiation • Stem cell

2.1 Introduction

Regenerative medicine is a new field of medicine with the aim to repair damaged organs and tissues after disease, traumas, or aging by using stem cells (Stocum 2001). Current research has mainly focused on the regenerative potential of

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embryonic stem (ES) cells, which have ethical limitations, and adult stem cells, which are less available and more difficult to grow in in vitro culture. However, despite their therapeutic potential, both cell types have specific limitations. Although adult stem cells can be directly isolated from the patient and are therefore immunologically compatible with the patient, they are generally difficult to isolate and grow in culture, and their recovery involves invasive interventions. In contrast, ES cells can proliferate rapidly in culture and differentiate into cells of all adult tissues; however, in addition to the need to resolve the ethical issues surrounding their use, they may lead to tumor formation after transplantation. Recently, the plasticity of amnion-derived cells was reported, and these cells have attracted much attention for their regenerative potential.

Amnion-derived stem cells, available in large quantities (as they can be retrieved from placentae at term), display clear characters of staminality, do not undergo oncogenic deviation, can differentiate into all three germ layers, and have no ethical concerns (Miki et al 2005). Moreover, since the placenta is where fetal–maternal immunotolerance is developed, these cells have immunomodulatory properties and appear to be well tolerated after allotransplantation. Collectively, these cells represent a promising candidate for use in regenerative medicine.

Despite these promising properties, the mechanisms that condition the staminality of these cells throughout expansion in vitro and differentiation are still largely unknown. Therefore, this research has been designed to investigate the properties of stemness of ovine amnion-derived stem cells and, in particular, of amniotic epithelial stem cells and their ability to undergo osteogenic differentiation after expansion in vitro. The data reported indicate that sheep amniotic epithelial cells (AEC) display interesting characteristics of staminality, and their osteogenic potential represents an alternative to bone marrow mesenchymal stem cells for the development of osteogenic regenerative strategies.

2.2 Materials and Methods

AECs were retrieved from the amniotic membranes of fetuses between 25 and 35 cm in length (i.e., at 3 months of development). After amniotic membrane isolation, the epithelial layer was mechanically peeled under a stereomicroscope and incubated in 0.25% trypsin in 0.5 mM Na-EDTA at 37°C for 20 min. The cells released were collected and seeded at a concentration of 3×10^3 cells/cm^2 in 175-cm^2 flasks in α-MEM supplemented with 20% fetal calf serum, 1% ultraglutamine, 1% penicillin/streptomycin, and 10 ng/ml epidermal growth factor. At 80% confluence, the cells were dissociated by 0.05% trypsin at 37°C for 5 min and plated again as described above. These procedures were repeated for 12 expansion passages. Aliquots of cells obtained from the 1st, 6th, and 12th expansion passages were used to evaluate the expression of surface adhesion markers (CD29 and CD49) and the nuclear stemness markers (Oct4, SOX2, Nanog, and TERT) by immunohistochemistry, and to investigate the level of cell-wide DNA methylation. The cells

were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature, washed with PBS, and permeabilized for 5 min with 0.1% Triton X-100 in PBS. After blocking the specific sites with 1% bovine serum albumin in PBS for 1 h, the cells were incubated with primary antibodies (anti-Oct4 1:200; anti-SOX2 1:200; anti-Nanog 1:1000; anti-TERT 1:250; anti-CD29 and anti-CD49 1:50; and anti-5-methyl cytosine 1:500) overnight at 4°C and were then exposed to secondary antibodies, Cy3 or Alexa fluor 488, conjugated at a final dilution of 1:500. Nuclei were identified with DAPI (1:5000).

The plasticity of AECs was assessed by culturing the cells in control medium (medium base), in osteogenic differentiation medium (DM, α-MEM with 50 μM ascorbic acid, 10 mM β-glycerol phosphate, and 0.2 μM dexamethasone), and in differentiation medium supplemented with estradiol (DMe, medium DM with 1 μg/ml 17β-estradiol). Osteogenic differentiation was first tested after 7 days of culture by evaluating the cell content of alkaline phosphatase (ALP), an early marker of differentiation, according to Thomas et al. (2007). The degree of culture mineralization of AECs was assessed at 7, 14, and 21 days of culture by evaluating the deposition of fluorescent calcein in the extracellular matrix with a Nikon Eclipse 600 microscope. For a semiquantitative analysis of matrix mineralization, fluorescent marker uptake was quantified using a Packard Fusion spectrophotometer (emission 500, excitation 540). The values obtained were normalized by dividing the fluorescence of cells cultured in differentiation medium by the basal fluorescence recorded in cells cultured in control medium. Cell culture mineralization was further confirmed by Alizarin staining at 14 and 21 days of culture. For this last analysis, the cells were washed with PBS, fixed in 4% paraformaldehyde for 10 min, and incubated in 2% Alizarin in 0.1 M sodium acetate, pH 4.2, for 90 min. The ALP values were compared using ANOVA, whereas values of calcein were compared using ANOVA with repeated measures considering significant values for $p < 0.05$.

2.3 Results

Immunocytochemistry showed the widely diffused presence of the stemness markers Oct4, Sox2, Nanog, and TERT in AEC, as well as the widespread expression of the adhesion surface molecules CD29 and CD49. The expression intensity of TERT and Sox2 was only slightly reduced after expansion in vitro, while Nanog and Oct4 expression remained constant. Moreover, the nucleus–cytoplasm distribution of TERT and Sox2 changed throughout the expansion. At the beginning of expansion, these markers were localized within the nucleus, similar to both Oct4 and Nanog, while in the more advanced phases of expansion, TERT and Sox2 were excluded from the nucleus and were segregated into the cytoplasm in 67.2% and 28% of cells, respectively. Conversely, the other markers preserved their nuclear localization in the cells throughout the expansion.

Table 2.1 Calcein uptake in AECs at the 1st, 6th, and 12th passages per days of culture in DM

	7 gg	14 gg	21 gg
1st pass.	8.42 ± 0.96[a]	8.02 ± 1.28	8.52 ± 0.98
6th pass.	4.45 ± 1.08	5.57 ± 0.84	8.22 ± 1.24
12th pass.	1.72 ± 0.57	2 ± 0.82	9.07 ± 1.26

[a]Values expressed as fluorescence units

Immediately after isolation, AECs did not present remarkable signs of global DNA methylation, while the presence of methyl cytosine was detected in cells at the 12th passage.

In all cells evaluated, no significant osteogenesis was recorded (increase in both ALP and calcein, and alizarin positivity) following a culture time of 21 days in control medium. Conversely, the use of inductive factors resulted in clear signs of osteogenic differentiation. Indeed, after 7 days of culture in osteoinductive medium, the quantity of ALP, expressed as absorbance, was 0.25 ± 0.01 in cells at the 1st passage and 0.17 ± 0.02 for controls ($p < 0.02$), 0.2 ± 0.01 at the 6th passage vs. 0.1 ± 0.02 in controls ($p < 0.05$), and 0.2 ± 0.02 at the 12th passage vs. 0.17 ± 0.03 in controls ($p > 0.05$). The addition of 17β-estradiol did not produce an increase in ALP activity. The values obtained in DMe were similar to those obtained in DM. Matrix mineralization, detected by calcein uptake after 7 days, was massive in cells at the 1st passage with large matrix droplets of extracellular mineralization accumulated on the cell monolayer, while calcein deposition was low in cells derived from the 6th and 12th passages. After 14 days, abundant calcein deposition was recorded in cells from the 1st passage, evident in the 6th passage, and barely detectable in the 12th passage. After 21 days, all evaluated cells expressed clear signs of matrix mineralization, with a higher signal in cells at the 1st passage. The addition of 17β-estradiol in culture medium improved the osteogenic transformation of AECs that remained evident in all cells types evaluated (calcein uptake in DMe 2–3 times higher than in DM). The quantification of the osteogenic process is reported in Table 2.1 and shows significant differences between control and DM, especially in the 1st passage ($p < 0.01$).

In cells cultured in DMe, the intensity of calcein deposition was always significantly higher than in DM ($p < 0.05$). Alizarin staining confirmed the diffuse process of mineralization in cells of the 1st passage, which was detectable after 14 days of culture and more evident after 21 days. By contrast, this staining procedure revealed clear signs of mineralization only after 21 days of culture in cells derived from the 6th and 12th passages but did not reveal any differences between DM and DMe.

2.4 Discussion

The present investigation describes the peculiar properties of AECs, which have a diffuse expression of adhesion molecules CD29 and CD49 that are likely responsible for the high plating efficiency of these cells and account for the quick

establishment of intercellular connections that result in the in vitro formation of three-dimensional spheroids as reported for human amniotic stem cells (Yen et al 2005). Moreover, the cells display a diffuse expression of stemness markers that is consistent with the rapid osteogenic differentiation that the cells undergo when exposed to inducing medium. Osteogenic differentiation can be achieved after prolonged expansion in vitro, and this process can be potentiated by simple molecules, such as 17β-estradiol, as described by others (Olivier et al. 2006; Kawaguchi et al 2005), and that could be used to improve the regenerative potential of the cells. Collectively, the data reported for the sheep model confirm that AECs represent a promising source of stem cells for the development of regenerative strategies. This investigation has demonstrated that after prolonged expansion, both the expression of stemness markers and osteogenic plasticity are reduced, thus suggesting that improved culture conditions are required whenever an extended amplification in vitro is necessary to obtain very large cell numbers.

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Chapter 3
NOS Immunoreactivity in the Reticular Groove of Lamb

M. Mazzoni, G. Petrosino, P. Clavenzani, F. Piccinno, V. Grasso, M. Serra, F. Rambaldi, and G. Lalatta-Costerbosa

Abstract Evidence suggests that the reticular groove (RG) may have an important role as the coordination center of reticulo-ruminal motility. The aim of this study was to investigate the presence and distribution of nitric oxide synthase (NOS) neurons and their phenotype in the RG of suckling lamb. Using double immunohistochemistry, many NOS neurons were found along the entire length of the RG and were more abundant in the floor than in the lips. They showed irregular profiles and often colocalized with galanin and dopamine β-hydroxylase but rarely with substance P and tyrosine hydroxylase. We never observed NOS neurons expressing neuropeptides normally found in intrinsic primary sensory neurons, such as calcitonin gene-related peptide, calbindin, IB4, and neurofilament 200 kDa. When compared to other districts, the enteric plexuses of the RG showed some peculiar aspects, such as the lack of both neurons in the submucosal plexus and typical sensory neurons.

Keywords Immunohistochemistry • Lamb • Nitric oxide synthase • Reticular groove

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3.1 Introduction

The gastrointestinal tract (GIT) is supplied by a collection of neurons embedded in its wall, referred to as the enteric nervous system (ENS). In most mammals, such enteric neurons are organized into two main ganglionated plexuses: the myenteric plexus (MP), lying between the longitudinal and circular muscle layers and mainly regulating muscle activity, and the submucosal plexus (SMP), located in the submucosal connective tissue and mainly regulating mucosal functions. So far, different subtypes of functionally distinct enteric neurons have been identified, that is, sensory neurons (or intrinsic primary afferent neurons, IPANs), motor neurons, and interneurons, organized into functional reflex circuits, which make the ENS capable of integrated reflex behavior without the involvement of the central nervous system (CNS). Many studies of the morpho-functional organization of the ENS have been conducted in the small and large intestines, while few studies have explored the reticular groove (RG) of bovine (Barahona et al. 1998; Teixeira et al. 1998) and sheep (Pfannkuche et al. 2003). The RG is a specialized region of reticulum consisting of a floor delimited by two lips that in suckling functions as a bypass between the esophagus and the abomasums to ensure the direct transport of milk via a vago-vagal reflex integrated in the gastric centers of the medulla oblongata. The neural mechanisms controlling RG movement are still largely unknown; however, compared to other forestomach (FS) FS compartments, the RG shows the greatest amount of vagal extrinsic innervation. Moreover, the RG is provided with the highest densities of ganglia and nerve fibers in the intramural plexuses with respect to other FS compartments (Teixeira et al. 1998); so, it has been suggested that RG intrinsic nerve pathways might be capable of coordinating the muscle activity of all the FS compartments (Gregory 1982). Pharmaco-physiological studies on the RG of calves have demonstrated that both noradrenaline (Denac et al. 1991) and acetylcholine (Barahona et al. 1998) play essential roles in the modulation of the activity and maintenance of muscle tone; in addition, a functional study involving esophageal groove strips revealed that nitric oxide (NO) plays a role in nonadrenergic noncholinergic (NANC) relaxation, which is significantly inhibited by incubation with inhibitors of NO synthesis. The aim of the present study was to investigate the presence and distribution of NO-producing neurons and their phenotype by means of double-immunofluorescence stains in cryostat sections in suckling lambs. Neuronal nitric oxide synthase (NOS) antiserum was used in association with antisera raised against neurochemical markers that have been used by other authors to identify adrenergic, sensory, inhibitory, and excitatory neurons and fibers such as tyrosine hydroxylase (TH), dopamine β-hydroxylase (DBH), substance P (SP), galanin (GAL), calcitonin gene-related peptide (CGRP), calbindin (CALB), isolectin B_4 (IB4), and neurofilament 200 kDa (NF).

3.2 Materials and Methods

The RG was dissected along its entire length in three suckling lambs, 1–3 days of age, and separated into proximal (the half next to the cardia) and distal (the half next to the reticulum–omasum orificium) parts. The specimens were fixed in 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.2) at 4°C for 24 h, then rinsed in phosphate-buffered saline (PBS), and stored at 4°C in PBS containing 30% sucrose and sodium azide (0.1%). The samples were frozen in isopentane cooled in liquid nitrogen. Transverse (i.e., perpendicular to the floor of the groove) cryosections (20 μm) were obtained along the entire length, mounted on poly-L-lysine-coated slides and submitted to double indirect immunohistochemical methods using the antibodies listed in Table 3.1. The sections were examined by a Zeiss Axioplan microscope equipped with appropriate filter cubes. The percentages of the NOS-immunoreactive (IR) neurons were calculated in nonconsecutive sections using NOS/NSE stain, and at least 100 cells were counted both in the lips and in the floor of each subject. For double-stained NOS/GAL and NOS/DBH, at least 50 neurons were counted for each subject both in the lips and in the floor. The values of the lip–floor junctions were included in the lip count.

3.3 Results

NSE-IR nerve fibers were observed along the entire length of the RG, and there was no obvious gradient of innervation between the proximal and distal parts. The neurons were generally clustered in ganglia varying in size; large ganglia (up to

Table 3.1 List of antibodies used in this study

Neuronal markers	Species	Code	Dilution	Supplier
NSE	Rabbit	N1516	1:5	DAKO
NOS	Mouse	Sc-5302	1:200	Santa Cruz
NOS	Rabbit	SA-227	1:300	Biomol
GAL	Rabbit	IHC 7153	1:400	Peninsula
DBH	Mouse	MAB308	1:200	Chemicon/Millipore
TH	Mouse	MONX10786	1:60	Monosan
SP	Rat	10-S15A	1:400	Fitzgerald
CGRP	Rabbit	C8198	1:800	Sigma-Aldrich
CALB	Rabbit	CB-38	1:800	Swant
NF200 kDa	Rabbit	N 4142	1:1000	Sigma-Aldrich
IB4	—	L2140	1 μg/mL	Sigma-Aldrich
Secondary antisera				
FITC-conjugated donkey anti-mouse IgG			1:100	Jackson
TRITC-conjugated donkey anti-rabbit IgG			1:500	Jackson
Alexa 594-conjugated donkey anti-rat IgG			1:400	Mol. Probes
FITC-conjugated streptavidin			1:100	Vector

50 neurons) were located in the MP and small ganglia (two to four neurons) or isolated neurons were observed intermingled with the longitudinal muscular fascicles of the lips. No neurons were found in the SMP. Both in the floor and in the lips, many NOS-IR fibers were observed along the entire length of the RG, parallel to the longitudinal axis of the muscle fascicles. Only a few NOS-IR fibers were located in the external longitudinal muscle layer of the floor and in the submucosa. NOS-IR neurons were located in the MP; they were more abundant in the floor than in the lips, and we rarely observed neurons located singly or grouped into small clusters of two to three cells among the muscle fascicles of the lips. NOS-IR neurons showed great variability in both size and morphology; they exhibited an irregular outline with either short, well-stained processes or an ovoid shape. In these neurons, a long process arising from the side opposite the nucleus could sometimes be seen without showing a given polarity. The neurons were more abundant in the floor than in the lips, and their number increased proceeding distally; however, their percentages were similar in the proximal and distal parts. Almost all ganglia in the floor contained NOS-IR neurons that were often arranged around the periphery of the ganglia, and some ganglia showed only NOS-IR neurons. Approximately 80% of the NOS-IR neurons in the floor coexpressed GAL; this percentage decreased in the lips (approximately 40%). Many neurons coexpressed DBH (from 32% to 58%) but rarely (less than 0.5%) TH and SP. We never observed NOS-IR neurons expressing CGRP, CALB, IB4, and NF; however, we never found neurons stained with neurochemical markers used to identify primary sensory neurons.

3.4 Discussion

This study shows a rich innervation in the RG of lambs, having a peculiar aspect with respect to the other districts of the same species and probably related to its particular function in suckling and adult animals. Contrary to what has been observed in the intestinal plexuses of both small and large mammals, including sheep (Lalatta-Costerbosa et al. 2007), no neurons were detected in the SMP. Similarly, SMP neurons, which are generally involved in secretion and vasodilation, are very few or absent in the esophagus and stomach of many mammals, including the sheep FSs (Yamamoto et al. 1995). Therefore, it is plausible to think that the myenteric ganglia in the lamb RG control mucosal functions. In the present study, many NOS-IR neurons coexpressed GAL. This neuropeptide is widely expressed in the CNS as well as in the ENS. In the GIT, the best-known effect of GAL is its ability to regulate GIT motility, either acting as a neurotransmitter by binding to three different G-protein-coupled receptors, all expressed in the GIT smooth muscle cells, or acting as a neuromodulator by both increasing and decreasing the release of neurohumoral substances (Gross and Pothoulakis 2007).

The extensive colocalization of NOS and GAL in the floor suggests that both neurochemical markers can act as inhibitory neuromodulators/neurotransmitters. The inhibitory role of NO on the muscle cells of the RG of bovines has been clearly demonstrated by Barahona et al. (1998) and may be supported by the lack of SP coexpression in nitrergic neurons in the present study. In fact, analogous to what has been observed in the GITs of many other mammals, it is plausible to hypothesize that SP has an excitatory role; furthermore, both acetylcholine and SP evoke contractions of isolated ruminal smooth muscle (Vassileva et al. 1978). The excitatory effect of SP has also been hypothesized by Pfannkuche et al. (2003) who found extensive colocalization of SP and acetylcholine in the RG of sheep, and whose contractile effects on the reticulum have been reported by Wong and McLeay (1988). In the present study, we rarely found neurons expressing CALB, CGRP, or IB4. Consequently, we can hypothesize that the sensory fibers of the RG may have an extrinsic origin coming from the spinal ganglia, in which both CGRP and NF neurons have been shown to project into the sheep duodenum and ileum (Mazzoni et al. 2010) or from the distal vagal ganglia or from primary sensory neurons located in other districts of the FS. Alternatively, it is tempting to speculate that, in the RG, intrinsic primary neurons, through which the enteric reflex is initiated, express unknown specific neurotransmitters, different from what has been observed in other regions of the same species.

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Chapter 4
Effect of Dry Period Length on NEFA and IGF-I Plasma Concentrations and Postpartum Ovarian Activity Resumption in Dairy Cows

M. Probo, A. Comin, A. Agazzi, I. De Amicis, A. Prandi, and F. Cairoli

Abstract The current study was carried out to examine the effects of dry period duration on NEFA and IGF-I plasma concentrations and postpartum ovarian activity resumption in dairy cows. Twenty-five pregnant Friesian cows (second and third lactation) were randomly assigned to one (standard dry period, 9 weeks, group C, $n = 12$) of two (short dry period, 5 weeks, group T, $n = 13$) treatments. Blood samples for NEFA and IGF-I analyses were collected once a week during the last 5 weeks prepartum and the first 14 weeks of lactation. Milk whey collection (twice/week) for progesterone analysis began 2 weeks after parturition and was used to detect ovarian activity resumption (at least three consecutive samples with P4 $\geq$ 300 pg/mL). The data obtained were analyzed by ANOVA for repeated data (mixed) and GLM of the SAS statistical package. Short dry periods reduced milk production (26.55 vs. 27.55 kg/day; $P \leq 0.01$), without modifying milk quality. The mean interval from calving to first postpartum cycle was shorter in group T than in group C (34.5 vs. 46.9 days, $P \leq 0.01$). No differences were found in NEFA plasma concentrations between groups either before or after calving, while IGF-I circulating concentrations were higher in group T than in group C during both the dry period and the first 14 weeks of lactation ($P \leq 0.01$). In conclusion, the reduction in the dry period had a positive impact on metabolic balance and time of postpartum resumption of ovarian activity.

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Keywords Dairy cow • Dry period • IGF-I • NEFA • Ovarian activity

Abbreviations

CP	Crude protein
dm	Dry matter
EE	Ether extract
IGF-I	Insulin-like growth factor I
NDF	Neutral detergent fiber
NEFA	Non-esterified fatty acids
NFC	Non fiber carbohydrate

4.1 Introduction

The choice of dry period length in dairy cows is mainly based on expectations of milk production; however, the effects on health and postpartum (PP) reproductive efficiency should not be underestimated. It is well known that the duration of dry periods has an influence on energy balance, which in turn affects reproductive efficiency (Watters et al. 2009). The first PP ovulation occurs 10–14 days after the energy balance has reached its nadir. Some authors (Rastani et al. 2005) have suggested that the reduction or the absence of a dry period results in a less-pronounced negative energy balance than does a traditional dry period.

Studies on the effects of a short dry period on PP reproductive performance have produced differing results (Gumen and Wiltbank 2005; Pezeshki et al. 2007). This study considers the impact of a short dry period on the PP resumption of ovarian cyclicity and on the energy balance in dairy cows by assessing NEFA and IGF-I plasma concentrations.

4.2 Materials and Methods

Twenty-five pregnant Friesian cows of second and third parity were divided into two groups (C, $n = 12$; T, $n = 13$) according to parity and lactation performance during the previous year (C: 25.5 kg/day, 3.6% fat, 3.3% protein, T: 26.5 kg/day, 3.6% fat, 3.15% protein). The dry period lasted traditionally (9 weeks) for group C and was short (5 weeks) for group T. Both experimental groups were fed the same ration (CP = 17.9% dm, EE = 3.2% dm, NDF = 41.3% dm, and NFC = 32.1% dm) containing: corn silage (40%), alfalfa hay (14.5%), alfalfa meal (14%), and

soybean meal (7.3%). Cows were fed 10 kg/cow/day during the dry period and 41 kg/cow/day during lactation.

Blood samples were collected weekly from the fifth week before parturition until the 14th week PP for the determination of plasma concentrations of NEFA (Accorsi et al. 2005) and IGF-I (Leman and Kinsella 1989; Devolder et al. 1993). Qualitative and quantitative characteristics of milk production were recorded for each cow. To evaluate the PP ovarian activity, milk whey concentrations of progesterone (P4) were determined twice per week starting from the third week after parturition (Comin et al. 2005).

The resumption of cyclicity was defined by the detection of three consecutive values of P4 $\geq$ 300 pg/mL (Comin et al. 2005). The data obtained were analyzed using ANOVA procedures for repeated data (mixed) and GLM of the SAS statistical package (SAS Institute 1994). Differences were considered significant for $P \leq 0.05$.

4.3 Results

The shortened dry period resulted in a significant decrease in milk production (26.55 vs. 27.55 kg/day, $P \leq 0.01$) without changes in fat (3.48 vs. 3.55%, $P = 0.19$) or protein (3.28 vs. 3.31%, $P = 0.52$) contents. Similarly, FCM (fat corrected milk) and FPCM (fat/protein corrected milk) production was significantly lower in group T than in group C ($P \leq 0.05$ and $P \leq 0.01$, respectively).

The NEFA profiles showed a similar trend in both groups, with increasing values from 340 and 358 μEq/L for group T and C, respectively. The maximum values were reached during the first week PP (957 and 908 μEq/L), followed by a gradual regression to concentrations of 318 (group T) and 294 μEq/L (group C) in the last sample (Fig. 4.3a). No significant differences ($P = 0.46$) were found between the two groups regarding NEFA plasma concentrations before or after parturition.

The IGF-I in group T declined to a nadir of 8.2 ng/mL in the first week PP, and then showed a positive trend until the end of the study (100.7 ng/mL). On the contrary, subjects in group C showed a decline in IGF-I levels from 58.9 to 9 ng/mL in the last weeks of pregnancy, and, after parturition, IGF-I returned to the initial levels (56.8 ng/mL) (Fig. 4.3b). The shortening of the dry period significantly raised the content of IGF-I in the final stages of the dry period and during early lactation ($P \leq 0.01$).

The resumption of ovarian activity, based on changes in milk whey P4, was recorded in 10 of 13 cows with short dry periods and 9 of 12 cows with traditional dry periods. Subjects in group T showed a significant decrease of the time interval required for the resumption of ovarian activity as compared to cows in group C (34.5 $\pm$ 3.55 vs. 46.9 $\pm$ 2.3 days, $P \leq 0.01$).

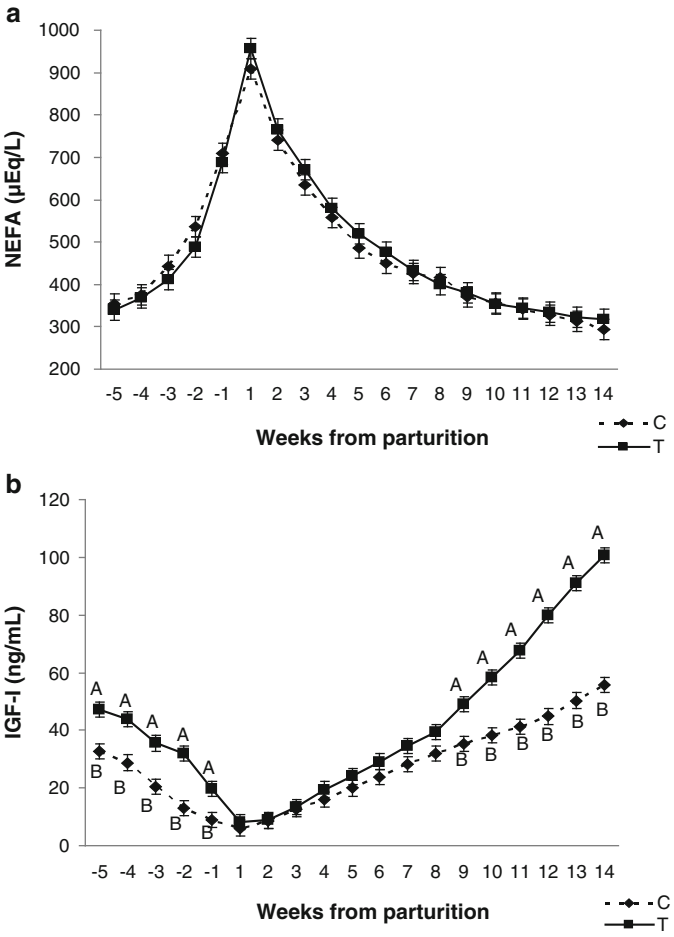

Fig. 4.1 (**a**) Mean (±SD) plasma concentrations of NEFA (µEq/L) in cows subjected to different dry period lengths. (**b**) Mean (±SD) plasma concentrations of IGF-I (ng/mL) in cows subjected to different dry period lengths. ($^{A,B}P \leq 0.01$)

4.4 Discussion

The reduction of the dry period to 5 weeks resulted in significant differences in the resumption of ovarian activity within 14 weeks PP in terms of time (34.5 vs. 46.9 days), but not numerically (76.9 vs. 75.0%). These results partially differ from data reported by Watters et al. (2009), who recorded, after a dry period of 34 days, a shorter interval between calving and first ovulation (35 vs. 43 days) and also a higher ovulation rate at 70 days of lactation (92 vs. 82%).

The pattern of NEFA plasma concentrations did not change significantly between the two treatment groups, which is in contrast to what was reported by

de Feu et al. (2009). The levels of IGF-I observed in group T were instead significantly higher during both the last part of the dry period and the first 14 weeks of lactation, displaying an improved energy balance as compared to the traditional dry period group.

Whole-milk production underwent a reduction of 4%, much lower than the 19% reported by de Feu et al. (2009), during the first 12 weeks of lactation. However, milk quality in terms of fat and protein content was not affected by the length of the dry period.

In conclusion, a reduction in the dry period resulted in a decrease in milk production, but improved the periparturient endocrine status, with a positive impact on both the metabolic balance and the time of postpartum resumption of ovarian activity.

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Chapter 5
Sheep Calcaneal Tendon Repair Subsequent to Amniotic-Derived Stem Cell Allotransplantation

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Abstract This preclinical study was performed to evaluate the regenerative capacities of vitro-expanded amniotic fluid stem cells (AFSCs) allotransplanted within experimentally induced lesions in ovine calcaneal tendons. Explanted samples were evaluated for tendon architecture, collagen composition, AFSC retrieval, cell proliferation, blood vessel organization, and leukocyte infiltration. Tendon healing and viable AFSCs were observed within the lesion site 30 days after transplantation. AFSCs differentiated into collagen-producing cells, stimulated collagen type I deposition and fiber alignment, and exerted positive effects on angiogenesis and inflammatory reactions. Thus, as AFSCs markedly improved tendon healing, they may be used to develop innovative therapies.

Keywords Allotransplantation • Amniotic fluid stem cells • Calcaneal tendon • Sheep

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5.1 Introduction

Tendinopathy is a very common condition; it is debilitating, painful, and usually caused by overwork. This medical condition affects patient quality of life and causes a sharp decline in locomotor skills. For this reason, tendinopathies are most common and feared in the world of sports, both in the medical field and in veterinary medicine (Dowling et al. 2000; Järvinen et al. 2001), where they are considered among the leading causes of reduced income in the horse racing industry. The concern associated with this medical condition is also increased by the impossibility of successfully treating this pathology using conventional treatments (physical and pharmacological therapies). For this reason, tendinopathies are among the diseases for which the use of regenerative cell therapy is viewed with increasing interest (Andres and Murrell 2008).

Stem cells are the basis of regenerative medicine, an innovative therapeutic approach that aims to repair tissues or organs damaged by congenital or acquired pathologies through the introduction of pluri/multi/unipotent cells (Bajada et al. 2008). Embryonic stem cells, for their biological characteristics (pluripotent cells), are the most promising cell source for therapeutic approaches. In fact, they proliferate rapidly and can differentiate into progenitor cells of different tissue types. However, strong ethical and legal constraints related to their use as well as their proven tumorigenic results considerably restrict their study and use in preclinical and clinical fields (Toda et al. 2007). In addition, the greater genomic stability and the absence of ethical limits of adult stem cells have discouraged further embryonic stem cell use (Smith et al. 2007). In fact, given the small numbers of adult stem cells, the difficulties encountered in their isolation and their low propensity to proliferate in culture do not fulfill the biological requirements necessary for subsequent stem cell transplantation in damaged tissues, in which these cells should restore full function. For this reason, there is increasing attention with regard to new sources of stem cells. Considerable interest exists in fetal stem cells, which combine ease of availability, lack of ethical limitations, and favorable biological characteristics, such as a high proliferative potential, high differentiation plasticity (multi-pluripotent), reduced tumorigenic drift, and high tolerance in recipient tissues (anti-inflammatory and immunomodulatory properties; De Coppi et al. 2007).

In this study, the regenerative properties of fetal amniotic fluid-derived stem cells (AFSCs) were evaluated in an experimentally injured tendon. This preclinical study was performed in sheep, which was chosen for easy availability of AFSCs (at slaughter) and similarities to the human morpho-functional skeletal muscle apparatus. The regenerative properties of the transplanted AFSC in the injured calcaneal tendon were evaluated by, combining histological and immunohistochemical (IHC) techniques, studying the architecture of the repaired tendon and verifying the incidence of inflammatory processes 15 and 30 days after stem cell allotransplantation. AFSC survival and integration into the injured tendon were monitored by vital lipophilic dye (PKH26) which allowed the identification of the transplanted

cells, even after a month. The reporter dye also allowed assessment of AFSC proliferative ability and documentation of their contribution to healing mechanisms by monitoring the expression of tissue-specific molecular markers (i.e., collagen types I and III).

5.2 Materials and Methods

AFSCs were isolated from sheep slaughtered between 60 and 80 days of pregnancy (fetal length: 18–25 cm). AFSCs were isolated from the amniotic fluid by centrifugation and cultured in 100-mm Petri dishes in the presence of α-MEM supplemented with 20% FCS, 2 mM L-glutamine, and 5 ng/ml bFGF2. Incubation at 38°C in 5% CO_2 continued until cells reached 70% confluence. The cells were then detached after enzyme treatment (trypsin-EDTA) and replated (2×10^4 cells/ml) as described previously.

AFSC phenotypic stability during culture was monitored by flow cytometry, studying the expression of hematopoietic (CD14, CD31, CD45, and CD58), adhesion (CD49f, CD29, and CD166), and stem cell (OCT , Sox 2, Nanog, TERT, and c-Kit) molecular markers.

Before transplantation, the cells were labeled by a 30-min coincubation with the lipophilic dye PKH26 (4×10^{-6} M) and then resuspended at a concentration of 2×10^6 cells in 20 μl PBS.

The experimental mechanical tendon defect was performed (a cylindrical lesion of 3-mm diameter × 3-mm depth) on the intermediate portion (5 cm proximal to the calcaneal tuberosity; Crovace et al. 2008) of both externalized calcaneal tendons after surgical incision. To this end, six 1-year-old Merinizzata sheep, weighing between 40 and 50 kg, were used; the surgery was performed after sedation of the animals. In the left tendon, the lesion site was covered with fibrin glue (Tissucol: Baxter Spa), while in the contralateral tendon, AFSCs were added to fibrin.

After 15 and 30 days, the animals were sacrificed; both calcaneal tendons were explanted and then processed for histological and IHC analyses. In particular, the portions of the tendons corresponding to the experimental lesions were immediately cryopreserved and completely cut into 10-μm sections. The tissue was analyzed by evaluating at least five sections in each internal, external, and middle portion of the lesion. On tissue sections, (1) the tissue architecture (hematoxylin/eosin, EE and Herovici, HE), (2) the composition of the extracellular matrix (IHC for collagen types I and III, Chemicon), (3) cell proliferation (IHC for the proliferation marker Ki-67, Santa Cruz), (4) the density and vascular organization (IHC for the endothelial marker, von Willebrand factor, vWF, Dako), and (5) the leukocyte infiltration (IHC for CD45, an antigen common to all leukocytes; Abd Serotec) were assessed. In the tendon graft, the presence and localization of PKH26-stained AFSCs were also assessed. All IHC staining was visualized using secondary anti-mouse Alexa Fluor488 (Molecular Probes) antibodies, while cell nuclei were labeled with DAPI

(Sigma). Finally, all treated sections were analyzed with the Axioscop2 plus fluorescence microscope (Zeiss) at low (×100) and high (×400) magnifications.

5.3 Results

5.3.1 Tendon Architecture

The histology of control (CTR) tendons showed, both at 15 and 30 days after explantation, deeply altered tissues characterized by hypercellularity, irregular tendon fibers, and composed mostly of collagen type III. In contrast, progressive tissue remodeling was observed in allotransplanted tendons. In this case, the tissue showed low cellularity, primarily represented by spindle-shaped cells in active proliferation (Ki-67 positive). The spindle-shaped cells (tenocyte-like cells), localized at lesion sites, were trapped between abundant extracellular matrix rich in collagen type I fibers, primarily oriented parallel to the longitudinal axis of the tendon.

5.3.2 AFSC Retrieval

At the lesion site, it was possible to retrieve AFSCs (PKH26-positive) at the periphery of the regenerating tendon even 30 days after transplantation. Most of the AFSCs appeared as flattened cells, located parallel to the longitudinal axis of the tendon fibers. Some of them colocalized with the proliferation marker Ki67, similar to neighboring cells. The transplanted AFSCs participated actively in the process of tissue repair by contributing directly to the neo-synthesis of collagen type I fibers.

5.3.3 Vascular Organization and Leukocyte Infiltration

In the CTR tendons, numerous small blood vessels (capillaries) were observed; they were disordered at the lesion site. On the contrary, the blood vessels found in AFSC-transplanted tendons were located parallel to the longitudinal axis of the tendon and between the neo-deposited extracellular matrix. A massive presence of leukocytes (CD45-immunopositive cells) was observed in control tendons, unlike allografted tendons where the leukocytes were found only inside the blood vessels.

5.4 Discussion

The present preclinical study allowed the documentation of AFSC regenerative capacity and their integration in experimentally injured tendon. In particular, according to the obtained results, it can be stated that AFSCs were able to stimulate tissue regeneration. In fact, 30 days after transplantation, the transplanted tendon resembled the architecture of a healthy tendon. The presence of new collagen type I fibers and their correct orientation, parallel to the longitudinal axis of the tendon, are the basis of full biomechanical recovery. AFSCs were also capable of promoting tissue repair by remaining viable and proliferating inside the lesion for 30 days, even in an allograft. These data confirm, also in the ovine model, that AFSC have low immunogenicity, which allows their use in allo- and xenotransplantation protocols (Niknejad et al. 2008). AFSCs most likely contribute to the stimulation of tissue repair mechanisms by promoting an indirect stimulatory effect on the progenitor cells present in the injured tissue. In fact, these cells showed active proliferation, especially at the lesion site that was subjected to cell transplantation. However, it is conceivable that AFSCs were also able to directly contribute to tendon regeneration by differentiating into tenocytes, as certain AFSCs recovered at the lesion site were able to produce collagen type I. Finally, among AFSCs regenerative mechanisms, a local anti-inflammatory effect cannot be excluded: modulating the angiogenic process and controlling inflammatory cell infiltration.

In conclusion, it can be stated that the integrated analysis of the results obtained could be used for the final optimization of all steps used to prepare the cells (cell source, cell passage number, cryopreservation protocol, in vitro stimulation, etc.) in relation to the therapeutic potential for immunogenicity and safety of integration. In fact, AFSCs could become a real and effective therapeutic tool, if new and more detailed information is acquired on their ability to differentiate and proliferate, the molecular mechanisms underlying these capacities, and their effectiveness after transplantation in terms of safety and treatment success. The identification of a rather complete array of parameters that reflect AFSCs regenerative potential will be used to optimize the procedures to obtain stable, controllable, and safe therapeutic cellular approaches for human cell therapy and clinical trials.

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Chapter 6
Role of Actin in Spermatozoa Function Through Biological Network Theory

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Abstract The effect of the actin polymerization inhibitor cytochalasin D during in vitro capacitation of boar spermatozoa was assessed. In particular, the acquisition of fertilizing ability and the main intracellular signaling pathways were analyzed. It was found that treated spermatozoa were unable to carry out capacitation, even if the biochemical analyses of the signaling machineries failed to elucidate any biochemical alterations. Using a computational model based on the biological networks theory and representing the molecular interactions involved in male gamete post-ejaculatory life, it was possible to explain these data: actin polymerization interacted with different subcellular compartments, acting as a coordinator of molecular pathways involved in sperm cell function.

Keywords Biological networks • Capacitation • Cytochalasin D • Spermatozoa

6.1 Introduction

The evolution of eukaryotes has been accompanied by an increase in the efficiency of signal transduction, produced by the spatial segregation of metabolic pathways and, at the same time, by an increase in their complexity. In fact, the same molecules can have different functions, depending on the subcellular compartment and the cell type within which they are expressed. For this reason, it is necessary, for the functional integration of different subcellular compartments, the presence of molecules that play a role within specific signaling pathways and that are delegated to the coordination of metabolic events.

The relevance of this topic in the study of physiology and pathology of cells, organs, and systems has led to the introduction of a new descriptive approach that

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can better represent cells and subcellular districts and that can analyze functional relationships between various signaling pathways. This method is based on the "biological networks theory" (Weng et al. 1999), which uses mathematical models and special software to represent cells as networks of molecules (the network nodes) that interact with each other, resulting in different interaction types (the links between nodes). Through subsequent statistical and topological analysis, it is possible to define the main parameters of each network and identify the key molecules of metabolic processes.

Recently, our research group developed a network to model the biochemical events occurring during the acquisition of fertilizing ability in boar spermatozoa (Bernabò et al. 2009). Such a cell type is particularly suitable for this analytical approach, since it is transcriptionally silent and, therefore, its protein content is almost constant. In addition, some biological events involving spermatozoa can be recreated and modulated in vitro, making possible the acquisition of molecular and biological information. In fact, it is possible to reproduce in vitro capacitation, the set of morpho-functional changes that allow the sperm to acquire full fertilizing power and induce acrosome reaction (AR); that is, the exocytosis of acrosome content in response to species-specific interactions with zona pellucida proteins (Abou-haila and Tulsiani 2009).

In this work, we use a computational model to aggregate and interpret the effects induced by inhibition of actin polymerization during capacitation and the AR promoted in vitro in boar spermatozoa.

6.2 Materials and Methods

Semen of three boars of proven fertility was used. Samples were divided into two different groups: control group spermatozoa (CTR) were cultured using validated conditions that promote capacitation (Maccarrone et al. 2005), while inhibition of actin polymerization was obtained by treating the sperm with a specific inhibitor, cytochalasin D (20 μM), from the moment of ejaculation and during the whole culture period, conducted as in the CTR group (CD). Both samples were incubated for 4 h at a final concentration of 1×10^8 spermatozoa/-ml. After incubation, the acquisition of fertilizing ability was verified, evaluating the incidence of AR in sperm exposed to solubilized zonae pellucidae (sZP) and staining spermatozoa with FITC-PSA (Barboni et al. 1995). In addition, the main signaling pathways were analyzed (a) acquisition of a pattern indicative of capacitated state by staining with chlortetracycline (CTC) (Mattioli et al. 1996); (b) pattern of tyrosine phosphorylation by western blotting; (c) localization of PLCγ1 in cytosolic and membrane fractions by western blotting and transmission microscopy (Spungin et al. 1995); and (d) concentration of intracellular calcium ($[Ca^{2+}]_i$), analyzed kinetically via confocal microscopy, using the fluorescent probe fluo-3-AM both before and after exposure to sZP. The biological results were included in the computational model recently validated by our group on mammalian sperm. This model was

developed using information drawn from the international literature (PubMed). Starting from these data, we created a database in Microsoft Office Excel 2007. For each metabolic event, we specified the original molecule, the target molecule, and the type of interaction (activation, inhibition, etc.). In addition, each molecule was assigned an attribute, depending on the specific intracellular localization within which it operates (cytosol, membrane, mitochondria, etc.). The network was upgraded using Cytoscape 2.6.1 software, in which molecules were arranged depending on the intracellular localization through the Cerebral plugin v.2.

6.3 Results

Biological results showed that inhibition of actin polymerization negatively affects the status of capacitation. In fact, exposure of in vitro capacitated sperm to sZP led to AR in 30% of CTR spermatozoa, while it caused the same reaction in only 10% of those treated with CD [$p < 0.01$ vs. CTR, analysis of variance (ANOVA)]. However, functional analyses performed on both groups of spermatozoa demonstrated no differences with regard to signal transduction pathways. In particular, CTC staining revealed a similar incidence of spermatozoa with fluorescence patterns typical of capacitated sperm in both CTR cells and those treated with CD (35.3 ± 4.3 vs. 36.7 ± 5.5, $p > 0.05$, ANOVA). In the same way, the kinetics and degree of protein tyrosine phosphorylation were similar. Furthermore, in vitro incubation promoted in both CTR sperm and CD cells a relocation of PLCγ1 with translocation of the enzyme from the cytoplasm to the sperm membrane. Finally, basal intracellular calcium levels were similar in CTR and CD sperm, and the rapid increase of [Ca^{2+}], induced by exposure to sZP, was detected in about 32% of analyzed cells, regardless of adopted culture conditions ($p > 0.05$ vs. CTR, ANOVA). To interpret these experimental results, an analysis of the computational model was performed, highlighting how actin polymerization is the node of the network that contacts the largest number of intracytoplasmic districts, with the specific biological role of integrating events taking place in different cellular compartments, such as cytosol, membrane, cytoskeleton, mitochondria, and acrosome (Fig. 6.1).

6.4 Discussion

The biological results obtained in this study showed that sperm incubated with CD under capacitating conditions negatively affected the acquisition of fertilizing power. In fact, the coincubation of sperm with solubilized zonae pellucidae, physiological agonists of AR, was unable to promote exocytosis of acrosomal contents. However, the study of the main signal transduction pathways involved in this process showed that they were unaltered by the presence of CD but were efficient. The functional interpretation of these data can be inferred by analyzing the

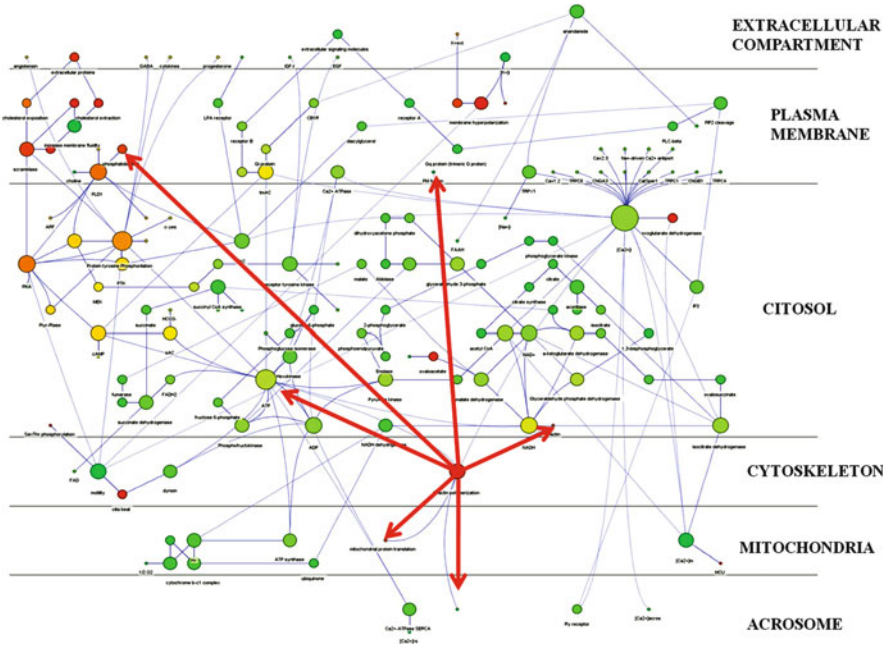

Fig. 6.1 Capacitation biological network. Actin polymerization (*red node*) interacts with (*red arrows*) all cellular compartments

computational model, representing molecules involved in the postejaculatory maturation of the male gamete and their interactions and taking into account different intracellular locations. Through this innovative approach, it was possible to highlight how actin polymerization is the crucial node in the interconnection of various subcellular compartments. This event appears to be related to all metabolically active compartments in the sperm during capacitation (i.e., cytosol, membrane, cytoskeleton, mitochondria, and acrosome). It is also interesting to note that the only cellular compartment that has no relationship with actin polymerization is the nucleus, which, in sperm, remains silent until the moment of fertilization. Thus, it is clear that actin polymerization likely plays a role in the coordination between the various metabolic pathways, harmonizing their operation. In the case of inhibition of actin polymerization (in our work after treatment with CD), in fact, the individual signal transduction pathways are normally activated, but the acquisition of fertilizing power is compromised by their lack of coherence. The identification of the cytoskeleton as a factor involved in the inside-out signaling of sperm appears to be important. First, it is in line with recent observations made in other cellular models (Shafrir et al. 2000), ascribing to this structure a leading role in the evolution and implementation of cellular signaling, independent on its mechanical function. It could then reveal new analytical and descriptive approaches for the physiology of the male gamete. Finally, it could suggest new prognostic or therapeutic strategies in reproductive medicine.

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Part II
Animal Pathology

Chapter 7
Staphylococcus aureus: Application of a Rapid Test for Molecular Typing of Strains Isolated from Bovine Mastitis

P. Cremonesi, V. Benedetti, G. Pisoni, P. Moroni, M. Luini, and B. Castiglioni

Abstract *Staphylococcus aureus* is the most important etiologic agent of contagious mastitis able to produce a number of virulence factors that may play important roles in the development of this disease. Recently, a rapid technique based on the amplification of the 16S–23S rRNA intergenic space (RS-PCR) was developed for *S. aureus* genotyping The aim of the present study was to characterize certain strains collected from North Italy herds to verify the presence of a specific genotype associated in Switzerland with severe clinical expression in the herd. In the 30 strains analyzed, our preliminary results showed the presence of four isolates similar to Switzerland genotype B and the possibility to link an electrophoretic profile to a specific pattern of virulence.

Keywords Genotyping • Mastitis • *Staphylococcus aureus*

7.1 Introduction

Staphylococcus aureus is one of the most important causes of clinical and subclinical bovine mastitis and is associated with great economic losses in dairy herds. Many *S. aureus* strains are able to produce a number of virulence factors that can destroy and lyse host cellular membranes and cause diseases. These factors include four distinct leukocidins, six hemolysins, over 20 different toxins, and three

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exfoliative exotoxins. Since the strain contagiousity seems to depend on virulence gene patterns, these factors could partially explain the different abilities some *S. aureus* strains have to colonize different host species or spread in the herd. As recently found in Italy (Benedetti et al. 2010), the antibiotic resistance of *mec*A-positive strains (methicillin-resistant *S. aureus*, MRSA) could be indirectly considered an additional virulence factor. In recent years, new methods with high discriminative powers (PFGE, spa-typing, and MLST) for *S. aureus* genotyping have been developed. These techniques have been used mainly for epidemiological purposes to differentiate between strains. In Switzerland, a recent *S. aureus* genotyping study used a relatively simple method based on the amplification of the 16S–23S rRNA intergenic spacer (RS-PCR); this technique is suitable for studies involving the analysis of numerous samples in a short time (Fournier et al. 2008). This assay made it possible to classify strains with high virulence and diffusivity into specific genotypic profiles. The aim of this study was to determine whether methicillin-resistant *S. aureus* (MRSA) or methicillin-sensitive *S. aureus* (MSSA) strains isolated from different Italian herds were included in the genetic profiles and virulence patterns similar to those documented in Switzerland.

7.2 Materials and Methods

7.2.1 Samples

Thirty *S. aureus* strains isolated from bovine mastitic milk samples were collected from 20 different northern Italian herds. DNA was extracted from 1 ml of culture broth (about 10^8–10^9 cfu/ml), following the instructions described in Cremonesi et al. (2006).

7.2.2 Analysis of Genes Associated with Virulence Factors

DNA was amplified to verify the presence of virulence-associated genes using primers and protocols described previously [*nuc*, *sea*, *sec*, *sed*, *seg*, *seh*, *sei*, *sej*, *sek*, and *sel* (Cremonesi et al. 2005); *seb* and *see* (Pinto et al. 2005; Monday and Bohach 1999); *coa*, *clf*A, *spa*, *tsst*, *eta*, and *etb* (Akineden et al. 2001); *leuk*E (Fournier et al. 2008); *Luk*S–*Luk*F/PV and *mecA* (McClure et al. 2006); *sak*, *fmtB*, *scn*, and *chp* (Sung et al. 2008); *LuK*E–*Luk*D and *Luk*M (Jarraud et al. 2002); and *cna* (Zecconi et al. 2006)].

7.2.3 RS-PCR Genotyping

For RS-PCR genotyping, the method described by Fournier et al. (2008) was used. PCR products were analyzed using an Agilent 2100 Bioanalyzer with a DNA 7500 LabChip kit (Agilent Technologies, Palo Alto, CA). For interpretation of the results, two patterns were considered different if two or more peaks of the electropherogram differed in size.

7.3 Results

7.3.1 Virulence-Factors

All strains were positive for coagulase (*coa*), termonuclease (*nuc*), and a membrane protein associated with antibiotic resistance (*fmt*B) gene, and were negative for genes involved in host-cell invasion (*eta*, *etb*, and *sak*). In the analysis of coagulase (*coa*) and protein A, the X region (*spa*) genes produced different-sized amplicons of 560 bp (6.6%), 640 bp (33.3%), 730 bp (36.6%), and 850 bp (23.5%), and of 150 bp (20%), 180 bp (30%), 250 bp (3.5%), 270 bp (13.3%), 290 bp (16.6%), and 320 bp (16.6%), respectively, which allowed for grouping of the isolates in clusters based on the fragments obtained. Among the strains, 11 (36.6%) were enterotoxigenic and coded for enterotoxins A, C, D, G, H, I, and J, with a prevalence of strains harboring the *seg* and *seh* genes and *sed* and *sej* genes (10%). Enterotoxins B, E, and L were never detected in the isolates. For the *luk*E (leukotoxin E) gene, 63.3% of the strains were positive, while 80% and 76.6% of the strains were positive for *cna* and clumping factor (*clf*A), respectively.

Half of the samples analyzed were MRSA (*mec*A positive) and one strain possessed the *tsst* gene that encodes a toxin responsible for toxic shock syndrome. Similarly, only one strain had the Panton-Valentine leukocidin (*Luk*S–*Luk*F/PV) gene. Eighteen out of the 30 (60%) strains possessed a gene coding for a new leukotoxin (*Luk*E–*Luk*D) and only three of these were positive for *Luk*M–*Luk*F/PV, which have been identified in strains isolated from bovine milk (Jarraud et al. 2002). Of the isolated strains, 3.3% and 6.6% were positive for *scn* and *chp*, respectively. These mobile elements are usually present in strains involved in human infections (Sung et al. 2008).

7.3.2 Genotyping

Analysis of the 16S–23S rRNA from 30 strains revealed 16 different profiles (Fig. 7.1). Of these profiles, four strains originating from three herds located in Piacenza, Como, and Sondrio had the same RS-PCR profile as the Swiss

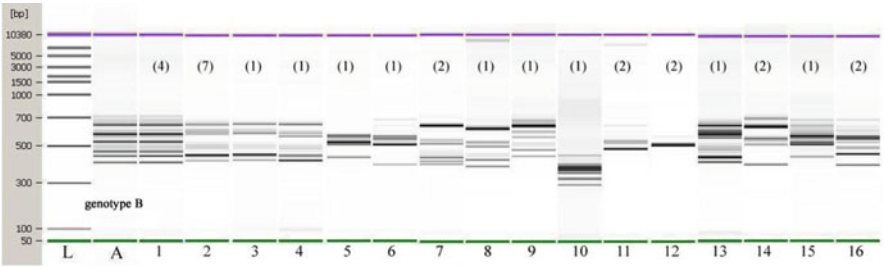

Fig. 7.1 Electrophoresis of the 16S–23S rRNA intergenic region PCR products by an Agilent Bioanalyzer. *L* ladder, *A* RS-PCR genotype B profile as described by Fournier et al. (2008); Lanes 1–16: RS-PCR profiles of samples analyzed in this preliminary study; (*N*) number of samples with the same profile

Table 7.1 Virulence genes of the strains with RS-PCR profiles 1 and 2

Profile	No. of strains	Enterotoxins (No. of strains)	*spa*	*coa*	*leukE*	*clf*A	*Luk*E–*Luk*D	*mec*A
1	4	*sec* (3), *sed* (3), *sej* (3)	>250	>640	4	4	4	0
2	7	*seg* (1), *sei* (1)	>100	>640	1	5	0	7

genotype B (Fournier et al. 2008), a genotype with high infectivity (Fig. 7.1: lane A, Swiss genotype; lane 1, samples analyzed in this study). Seven other strains isolated from four different herds near Lodi had the same profile shown in Fig. 7.1, lane 2, with the virulence characteristics that are summarized in Table 7.1. The remaining 14 profiles shown in Fig. 7.1 (lanes 3–16) resulted from the analysis of one or two strains isolated from samples collected at different times in the same herd.

7.4 Conclusions

The results obtained from this study revealed that the RS-PCR genotyping that was previously tested in Switzerland herds can be used in Italy as a rapid test that allows for grouping of heterogeneous *S. aureus* strains into defined subtypes. According to Fournier et al. (2008), the presence of a *spa* gene with a fragment size greater than 250 bp and a specific combination of genes encoding enterotoxins and leukotoxins is associated with virulence and pathogenic properties of the strain. This preliminary study has demonstrated the presence of genotype B strains in northern Italian herds. Additionally, unlike strains belonging to the RS-PCR profile type, none of the genotype B strains isolated in this study was *mec*A positive. The association of infectivity with high-profile resistance to antibiotics would require greater action to control disease and a strategic plan for the treatment of positive animals.

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Chapter 8
Epidemiological and Biomolecular Updates on *Encephalitozoon cuniculi* in Lagomorpha of Sardinia (Italy)

A.P. Pipia, M. Giobbe, P. Mula, A. Varcasia, G. Sanna, J. Walochnik, A. Lavazza, and A. Scala

Abstract A survey was carried out to determine the prevalence of *Encephalitozoon* spp. in lagomorphs of Sardinia (Italy) at nine industrial rabbit farms (*Oryctogalus cuniculus*) and in two breedings of hares (*Lepus capensis mediterraneus*). A total of 378 slaughtered rabbits (meat rabbits and breeders) and 45 living hares were examined. The survey was carried out including three different approaches: serological, pathological, and biomolecular. Sera obtained after centrifugation were frozen at −18°C until examination by carbon immunoassay (Medicago, Uppsala, Sweden). Six out of the nine rabbit farms were found to be serologically positive for *E. cuniculi* (66.7%), while the number of seropositive rabbits was 68 of 378 (18.5%). All hare sera tested negative. In all slaughtered rabbits, macroscopic renal lesions, presumably attributable to infection with *E. cuniculi*, were detected. Gross lesions were found in 62 out of the 378 individuals examined (16.4%). DNA was extracted from 22 samples that were positive for microsporidia and subjected to sequencing and genotyping. All isolates investigated showed 100% homology to *E. cuniculi*.

Keywords CIA Test • *Encephalitozoon cuniculi* • Lagomorphs • PCR • Sardinia

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Abbreviations

CIA Carbon immunoassay test
PCR Polymerase chain reaction
MR Rabbit meat
OB Old breeder

8.1 Introduction

Encephalitozoon spp. are obligate intracellular microsporidia that occur in a wide range of hosts; *E. cuniculi* affects mainly rabbits. In the European hare, infection by *E. intestinalis* and *E. hellem* has been described only once in Belgium (De Bosschere et al. 2007). Infection occurs either by ingestion or inhalation of spores or by transplacental transmission from mother to fetus (Harcourt-Brown 2004).

Encephalitozoon spp. are also considered opportunistic pathogens in humans with acquired immunodeficiency syndrome (AIDS) or immunosuppression (Zender et al. 1989; Deplazes et al. 1996; Wasson and Peper 2000). Three species have been revealed as human pathogens: *E. cuniculi*, *E. hellem*, and *E. intestinalis*, morphologically indistinguishable from each other. In lagomorphs, *E. cuniculi* infection is mainly diagnosed serologically.

However, the presence of antibodies only indicates chronic infections with *E. cuniculi*, and in vivo diagnosis in rabbits is rather difficult. Diagnosis is usually achieved by a combination of clinical, neurological, and ophthalmologic examination, supported by serological tests and differential diagnosis with other diseases.

Detection of *E. cuniculi* by PCR appears to be a very sensitive and specific method to confirm infection. However, this technique is not yet widely accepted (or well established) for diagnostics in rabbits with alleged encephalitozoonosis (Künzel et al. 2008). This is probably attributable to the fact that, in live animals, PCR appears to be too laborious and economically expensive for routine practice; thus, it is used mainly for research purposes in slaughtered rabbits.

The goal of the present survey was to investigate the presence of *E. cuniculi* in the breeding of rabbits and hares in Sardinia through a multidisciplinary approach including serology, histopathology, and biomolecular techniques.

8.2 Materials and Methods

The survey was carried out in Sardinia (Italy) at nine rabbit farms (*Oryctogalus cuniculus*) and two herds of hares (*Lepus capensis mediterraneus*). A total of 378 slaughtered rabbits were examined serologically, of which 261 were animals for meat (MR) and 117 were breeders at the end of their careers (OB) of 3 years. All animals were of the California/New Zealand genotype/line or their hybrids, while

the 45 hares came from two farms in the South of the island managed by the Ente Foreste of Sardinia. The survey was developed following three main protocols: serological, histopathological, and biomolecular.

Blood was withdrawn individually prior to butchery, and sera obtained after centrifugation were frozen at −18°C until serology tests were carried out with the CIA (Medicago, Uppsala, Sweden).

All slaughtered rabbits were then inspected for macroscopic renal lesions presumably attributable to *E. cuniculi*, and a valued score was used to classify them: 0 = no lesions, 1 = localized small white spots, 2 = widespread presence of small white spots, 3 = localized scarring, and 4 = wrinkle kidney.

From 22 out of 94 samples for biomolecular and histopathological surveys, DNA was extracted with a commercial kit (High Pure Template Purification Kit, Roche), PCR was performed according to the protocol developed by Valencakova et al. (2005), and sequencing was carried out on an AB automated sequencer (Applied Biosystems, Vienna, Austria).

8.3 Results

Six out of nine breedings monitored were serologically positive to *Encephalitozoon* spp. (66.7%), while all hares' sera tested were negative.

The prevalence for positive farms ranged from 0% (Farm 1) to 24% (Farm 2), 25% (Farm 7), and 80% (Farm 3), respectively, in MR and OB animals. MR prevalences observed in the positive farms (No. 5) differed statistically significantly (χ^2 = with 4 degrees of freedom = 10.32, $P = 0.035$), while differences were not statistically significant in OB (χ^2 with 4 degrees of freedom = 5.87, $P = 0.208$).

Of sera examined, 18.5% (68/378) were positive, in particular 5% (13/261) of MR and 47% (55/117) of OB. The differences between the total prevalence of OB and MR were statistically significant (χ^2 Yates corrected = 93.89, $P < 0.0001$).

The kidneys with macroscopic lesions were 16.4% (62/378) stratified according to the following classes of scores: score 1 = 6.6% (25/378), score 2 = 5.5% (21/378), score 3 = 2.6% (10/378), and score 4 = 1.6% (6/378). The kidneys of seropositive animals with the macroscopic lesions were 39.7% (27/68). The difference between the total percentage of kidneys with lesions and the seropositive animals was highly significant ($\chi^2 = 23.03$, $P < 0.0001$). Correlation between the lesion scores and seropositives was positive (Pearson correlation = 0.211) and statistically significant ($P < 0.0001$).

Of 22 positive samples examined, eight (36.4%) were identified as *Encephalitozoon* spp. by DNA sequencing, of which six (27.3%) were also positive by serological testing, while one sample was negative, and one was not tested serologically. Sequences showed 100% homology to *E. cuniculi*.

8.4 Discussion

The present survey confirmed that *E. cuniculi* is a widespread parasite in Sardinian industrial rabbits, as verified by molecular biology. From our study, it appears that OBs seem to act as reservoirs of the parasite, in which the seroprevalence rates reach values that must not be underestimated. The nondetection in hares is in line with the national and international situation, given that this species has never been found positive for *E. cuniculi*.

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Chapter 9
Antibiotic Resistance Profiles in Relation to Virulence Factors and Phylogenetic Groups of Uropathogenic *Escherichia coli* Isolated from Dogs and Cats

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Abstract In this study, we assessed the relationship between antibiotic-resistance profiles, virulence genotype, and phylogenetic group within a collection of *Escherichia coli* obtained from dogs and cats with urinary tract infection (UTI). Forty uropathogenic *E. coli* (UPEC) strains isolated from dogs ($n = 30$) and cats ($n = 10$), formerly analyzed for virulence factors (VFs) and phylogenetic group, were tested to detect antibiotic resistance for gentamicin (GM), trimethoprim–sulfamethoxazole (SXT), nitrofurantoin (NT), enrofloxacin (ENO), cephalothin (CF), cephoperazone (CFP), cefovecin (CVN), piperacillin (PIP), and amoxicillin–clavulanic acid (AMC). A large number of isolates were resistant to cephalosporins, especially to third-generation drugs, while the lowest level of resistance was found to SXT. No statistically significant results were obtained ($P > 0.05$). Concerning antimicrobial resistance associated with VFs, only gene *iutA* showed an association trend with multidrug resistance (MDR; $P = 0.055$). Resistant strains were distributed in all phylogenetic groups (57%, B2; 43%, non-B2), whereas *E. coli* isolates susceptible to all antibiotics tested were associated with groups B2 (90%) and D (10%).

Keywords Antimicrobial resistance • Cats • Dogs • *Escherichia coli* • Phylogroup • Urine • Urine virulence factors

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Abbreviations

MDR	Multidrug-resistance
PCR	Polymerase chain reaction
UPEC	Uropathogenic *E. coli*
UTI	Urinary tract infection
VFs	Virulence factors

9.1 Introduction

Escherichia coli is a frequent cause of urinary tract infection (UTI) in humans and in companion animals such as dogs and cats. This disease, often associated with urogenital diseases, such as cystitis, nephritis, and prostatitis (Wilson et al. 1988), is a serious clinical problem, which is difficult to solve, and is associated with antimicrobial resistance, the presence of virulence factors (VFs), and tissue colonization. The latter are all factors contributing, in some cases, to persistence of infection. The majority of uropathogenic *E. coli* (UPEC) strains produce specific VFs. Most UPEC strains are able to infect noncompromised hosts and, contrary to intestinal strains, belong prevalently to phylogenetic group B2 and less frequently to group D. In humans, the UPEC strains that cause UTI usually do not present high antibiotic resistance and are associated with the presence of an elevated number of VFs (Johnson et al. 2003).

The aim of the present study was to assess the relationship between antibiotic-resistance profiles, virulence genotype, and phylogenetic groups within a collection of *E. coli* strains isolated from dogs and cats with UTI.

9.2 Material and Methods

A total of 40 *E. coli* isolates from dogs ($n = 30$) and cats ($n = 10$) with cystitis were used. The urine samples were cultured on MacConkey agar (Oxoid, Basingstoke, Hampshire, England), and lactose-fermenting, indole-positive colonies were evaluated by the BBL Crystal test (Becton, Dickinson and Company, USA). The isolates had been previously investigated for eight virulence markers (*fimA*, *papC*, *cdt*, *sfa*, *afa*, *iutA*, *hlyA*, and *cnf1*) by polymerase chain reaction (PCR) and assigned to phylogenetic groups (A, B1, B2, and D) by multiplex-PCR (Tramuta et al. 2011). Antibiotic-resistance profiles were performed using the disk diffusion method, following the National Committee for Clinical Laboratory Standards (2001). All strains were tested with antimicrobial agents on the basis of their importance for the treatment of urinary infections on companion animals and belonging to different classes of chemotherapeutics: gentamicin (GM), trimethoprim–sulfamethoxazole (SXT), nitrofurantoin (NT), enrofloxacin (ENO),

cephalothin (CF), cephoperazone (CFP), cefovecin (CVN), piperacillin (PIP), and amoxicillin–clavulanic acid (AMC). Statistical analysis was determined using Fisher's exact probability test, considering p values less than 0.05 as statistically significant and less than 0.01 as highly significant.

9.3 Results

Distribution of virulence markers was as follows: *fimA* 85%, *sfa* 57.5%, *cnf1* 52.5%, *papC* 37.5%, *iutA* 37.5%, *hlyA* 27.5%, *cdt* 7.5%, and *afa* 2.5%. At least one of the virulence-associated genes was present in all strains; in particular, six samples carried only one of the target genes and no sample had more than six VFs. As to the phylogenetic distribution, 65% of the isolates belonged to group B2, 10% to group D, 15% to B1, and 10% belonged to group A. *E. coli* strains with at least three VFs belonged, on the whole, to phylogroup B2 (90.5%), and isolates with a number less than three VFs were distributed into all phylogenetic groups. *FimA* and *iut*A genes were detected in all groups with different percentages, while *papC*, *hlyA*, *cdt*, and *afa* were only found in phylogenetic group B2; in particular, *papC* and *hlyA* were significantly associated with B2 ($p < 0.05$). *Sfa* was mainly detected in group B2, and *cnf*1 was distributed into all groups, except group D. The relationship between the *hlyA* gene, considered a genetic marker for *E. coli* virulence, and others factors was evaluated, obtaining a highly significant ($p < 0.001$) association between *hlyA* and *sfa*, *papC*, and *cnf1*.

The frequency of antimicrobial-resistant *E. coli* is shown in Fig. 9.1. No antibiotic tested was effective on all strains. Fifty-five percent of the isolates showed resistance to at least three antimicrobial drugs and were designated as being multidrug resistant (MDR), while 25% of the strains were susceptible to all tested antimicrobial agents.

A large number of isolates were resistant to cephalosporins, specifically to the third-generation drugs, while the lowest level of resistance was found to SXT.

E. coli strains isolated from dogs and cats showed similar antimicrobial profiles, and no statistically significant differences between two species were observed, except for cefovecin ($p < 0.05$).

Antibiotic-resistant isolates belonged to all phylogenetic groups (57% B2; 43% non-B2), while susceptible strains were associated almost exclusively in group B2 (90%) with a smaller percentage in group D (10%; Table 9.1).

Only SXT-resistant strains showed a highly statistically significant association with phylogenetic groups A and B1 ($p < 0.01$), whereas CF-resistant strains only showed a trend of association with these phylogenetic groups. Concerning the VF relationship with antimicrobial resistance, no significant results were obtained ($p > 0.05$); only gene *iutA* showed a trend of association with MDR ($p = 0.055$). The virulence score also obtained from susceptible and antibiotic-resistant strains showed no significant differences (Table 9.1).

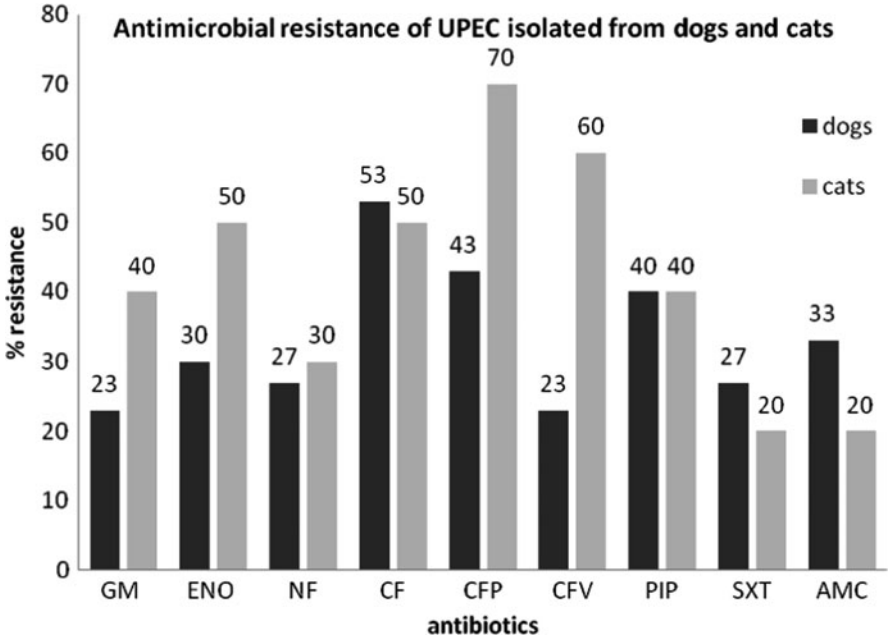

Fig. 9.1 Frequency of the antimicrobial resistances (%) in UPEC strains isolated from dogs and cats

Table 9.1 Distribution of virulence factors (VFs) and phylogenetic groups in relation to antibiotic-resistance profiles of *E. coli* strains ($n = 40$) examined

	Gene	Susceptible strains ($n = 10$)	Resistant strains ($n = 30$)
VFs	*papC*	4 (40%)	11 (37%)
	cnf1	6 (60%)	15 (50%)
	sfa	6 (60%)	17 (57%)
	fimA	9 (90%)	25 (83%)
	iutA	2 (20%)	13 (43%)
	hlyA	3 (30%)	8 (27%)
	cdt	0	3 (10%)
	afa	0	1 (3%)
Score (median)	–	2.5	3
Phylogroup	*B2*	9 (90%)	17 (57%)
	Non-B2[a]	1 (10%)	13 (43%)

[a]Non-B2: phylogroups A, B1, and D

9.4 Discussion

In the present study, we investigated the relationship between antimicrobial resistance, virulence genotype, and phylogenetic background for UPEC strains isolated from dogs and cats.

Similar genotypic and phenotypic profiles were found for both species, confirming that there was a common source of antimicrobial-resistant *E. coli*. The prevalence of VFs is in agreement with other studies conducted on human- and animal-origin strains (Féria et al. 2000; Johnson et al. 2004). As to the association between VFs and phylogenetic background, *hlyA* was only present in phylogroup B2. The latter may indicate that *hly* presence is associated with high pathogenicity of the strains, not only owing to the toxin produced but also because the strains harboring the gene may also be carriers of other VFs. The strains examined belong prevalently to group B2 with a discrete virulence score, suggesting that these strains are more virulent than non-B2 strains. In accordance with other authors (Johnson et al. 2004; Rijavec et al. 2006), we observed a strong correlation between antibiotic susceptibility and phylogroup B2, while we found no relationship between antibiotic resistance and low occurrence of VFs (Johnson et al. 2004). In conclusion, our data confirm that antibiotic-resistant UPEC strains continue to increase in cats and dogs, thus suggesting the need for the careful use of antimicrobials to treat UTIs in companion animals.

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Part III
Pharmacology and Clinical Science

Chapter 10
Effects of Ambient Temperature on Calf Welfare Parameters During Long-Road Transportation

D. Bernardini, G. Gerardi, D. Degani, C. Guglielmini, and S. Segato

Abstract In the tested ambient temperature range (i.e., between −7 and 30°C), it was asserted that calves' physical regulatory system of temperature homeostasis regularly works, particularly under ambient winter conditions. In summer, only ambient temperatures near 31°C could lead to reduced operation of the thermoregulatory center with the presence of clinical signs. This finding is strengthened by the correlation between ambient and rectal temperatures ($R^2 = 0.57$; $P < 0.001$); rectal temperature appears to remain constant within an ambient temperature range between 8°C and 28°C. Furthermore, ambient temperatures lower than −7°C cause a slight reduction in body temperature, while values higher than 29°C result in a more than proportional physical temperature increase, which are better interpolated using a polynomial equation.

Keywords Animal welfare • Calves • Ethology • Transportation

Abbreviations

AT, RT, and ST	Ambient, rectal, and subcutaneous temperatures
BW	Body weight
HR	Heart rate

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10.1 Introduction

The twenty-first century is marked both by a free exchange of goods and by an intense flow of people, which increase the need to market living animals (Swanson and Morrow-Tesch 2001). Regarding this complex phenomenon, the opinion of farmers, traders, and consumers sometimes does not agree. However, animal handling can address this issue, assuring a lack of stress and increased health during animal transport and allowing optimal meat-quality traits. Animal welfare provides the moral duty of respecting the animals and their sensitivities (sentient animals) using more suitable rearing systems (Broom 1998, 2003). Animal transport encompasses stressors that act with different intensities during the journey and depend on individual reactivities toward each stressor (Fazio and Ferlazzo 2003). Thermic stress should be investigated to determine whether an animal's adaptation remains within normal physiological limits or markedly involves the thermoregulatory system, causing simultaneous changes in welfare parameters (Verga 1994), leading to the exhaustion phase of the general adaptation syndrome (GAS) (Selye 1946). This study examines routine animal haulages to evaluate the effects of ambient temperature (seasonal effects) on clinical, physiological, behavioral, and productive welfare indicators (Grandin 1997). For this purpose, two similar long-road transports (transport effect; 9 h journey, 1 h stop, 9 h journey, and further 2 h journey to the destination farm) were made in different ambient temperatures: summer (July) and winter (January).

10.2 Materials and Methods

Two calf transports were carried out from Poland (Warszawa) to Italy (Padova; 1,400 km) in July and January. All transported calves ($n = 180$ in summer and $n = 192$ in winter) were chosen from Polish farms and transferred to a transit center (Wolborz, Poland) where they stayed for 30–34 h before leaving for rearing at Italian farms. The loading density was equal to 0.55 m^2 per head, allowing the calves to lie down suitably. In both haulages, the calves were monitored from the Polish farms to the farm of destination and for some time afterward. The Holstein Frisian calves (30–45 days and 65–80 kg) were clinically healthy during the experimental period. Their RTs were measured. Electrocardiogram electrodes, which were placed on the skin, and a subcutaneous electrode allowed monitoring of ECG, ST, and the movement of the calves by a Dispositivo Medico Multi – Parametrico in Radio Frequenza DEP (Wireless Sensor Networks S.r.l., Monza, Italy). The calves were weighed and fed before loading. During both transports, each of the seven sampled calves was placed in each box of the three loading platforms of the truck. The transports were made by the same truck, drivers, roadways, and stops; the truck had all requirements provided for by law. The summer and winter AT (Data Logger 32 – Siemens) correlated to the loading

platform temperature (mean of seven sensors) and to the ST. Relative humidity was recorded only at departure and arrival. BW was measured at the Polish farms, in the transit center before departure, and after arrival in Italy.

BW data were analyzed according to a linear model that considered the seasonal and transport (repeated measurements) effects. The influence of the AT and truck temperature on RT and ST was investigated by linear regression to determine significance. After evaluation of the correlation matrix, a nonlinear regression was made between AT and RT, which showed an increased value of the coefficient of determination (R^2).

10.3 Results

No transported calves showed any trauma or cutaneous injury; no primary or secondary skin lesions were observed. In July, an AT of 14°C was observed at departure from Poland and of 29°C after arrival in Italy with 65% and 55% relative humidity, respectively. In January, ATs of −5°C at departure and 6°C after arrival were observed with 89% and 72% relative humidity, respectively (Fig. 10.1a). Despite these marked variations, the inside truck temperature slightly changed (Fig. 10.1b). In these climatic conditions, the calves maintained an RT control in both transports by superficial (skin thermic receptors) and deep thermoregulation (hypothalamic centers connected with the regulation of respiration, circulation, and other essential functions) without any pathological signs. In fact, RT was 38.8–39.9°C in the summer and 38.3–39.6°C in the winter (Fig. 10.2a) within the normality range of 38.5–39.5°C (Radostis et al. 2007). The greatest increases in RT were observed after unloading. A significant linear correlation was found between AT and RT ($R^2 = 0.57$; $P < 0.001$), but not between AT and ST ($R^2 = 0.19$; $P > 0.10$) as reported in Fig. 10.2a, b. ST showed a slight variation between 38.4 and 38.9°C in July, and between 38.1 and 38.7°C in January (Fig. 10.3a).

Before departure, the calves showed a decrease in BW equal to 7.0% in July and 4.9% in January compared with values recorded at the Polish farm. After arrival in

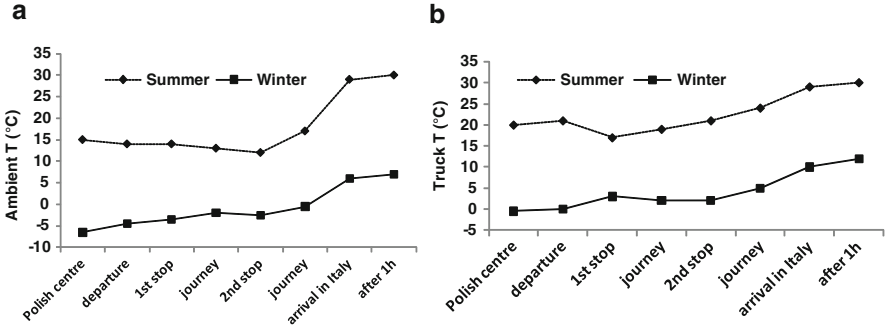

Fig. 10.1 AT (*left*) (**a**) and truck temperature (*right*) (**b**) according to transport schedule

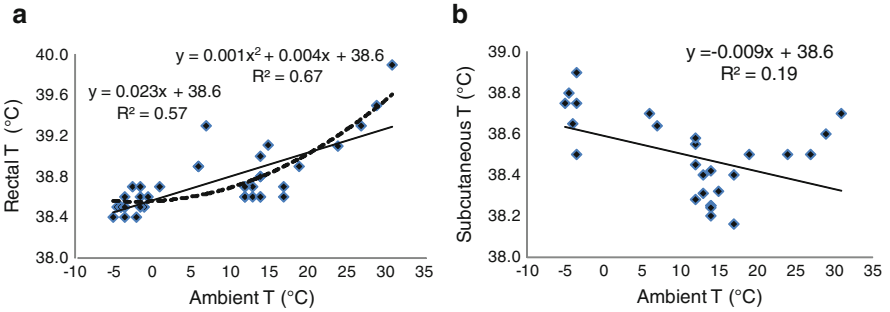

Fig. 10.2 Correlation between RT (*left*) (**a**) or ST (*right*) (**b**) and AT during the journey

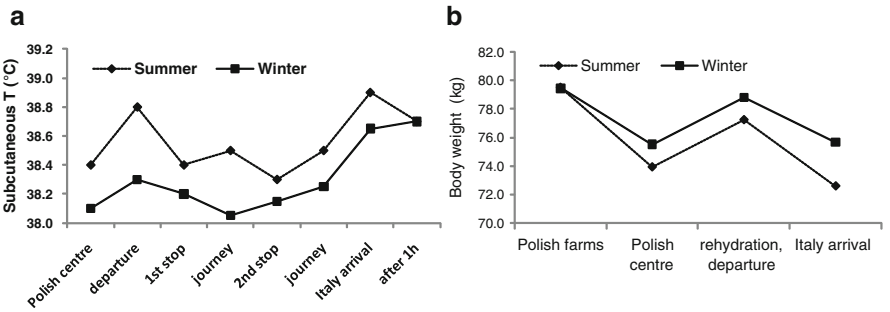

Fig. 10.3 ST (*left*) (**a**) and BW (*right*) (**b**) according to transport schedule

Italy, they showed a weight loss equal to 6.0% in July and 4.0% in January compared with before departure (Fig. 10.3b). During both seasons at the transit center after weighing, the calves were fed 5–6 L milk, increasing their BW by 4.4%. Therefore, the weight loss from the Polish farm to the Italian farm was equal to 8.7% (6.9 kg) in the summer and 4.8% (3.8 kg) in the winter. Afterward, a progressive increase in BW was seen within 24 h of arrival at the Italian farm. Seasonal effects had a significant influence on BW; likewise, transport effects were significant, showing a lower weight loss during the winter journey. ECGs revealed higher HR during loading and unloading, an increase of 31% and 26%, respectively, which are slightly higher than the normal range of 100–120 (Radostis et al. 2007). As the polarity of the deflections differed between calves, the P waves in DII were positive in some calves and negative in others. The mean electric axis varied from 60 to 180°.

10.4 Discussion

Twenty-hour road haulages, if carried out with suitable handling, personnel, and trucks, may lead to discomfort but not to stress because only the phases of alarm and adaptation, but not the phase of exhaustion of the GAS, should be expressed

(Selye 1976). The wide range of AT during both transports activated adaptation in the calves that was controlled by efficient thermoregulation, assuring physiological homeothermia without the appearance of thermic stress. Within the tested thermic range, thermoregulation functioned properly, particularly in winter conditions. In summer, only AT near 30°C reduced the function of the thermoregulatory center with the concomitant appearance of clinical signs. Comparing summer and winter temperatures, a moderate linear correlation between AT and RT was observed, indicating that RT kept itself almost steady. The decrease in BW during the journeys could be due to fluid losses that were greater in the summer. In comparison with BW at the Polish farms, the weight loss recorded before departure could be ascribed to massive feeding given by the Polish farmers to achieve a greater sale price. Even though the calves did not show, during either transport, any clinical signs and only slight dehydration, it is important to prevent possible electrolyte and mineral losses by early supplementation during the first hours after arrival. Ethological anomalies, signals of psychophysical discomfort (stereotypes), were seen only at limited times without appearing obsessive and/or maniacal. During both transports, at the first stop, lying-down calves stood up within 3–4 min. During the journey, they exhibited cross-licking, cross-sucking, and self-grooming. After departure and for the whole length of the transport, the calves placed themselves with their cranial/caudal axis perpendicular to the driving direction. The animals showed discomfort during loading and unloading; furthermore, less psycho-physical discomfort was observed in the calves placed in the lower boxes of the truck compared with the upper ones. The changes found in the ECGs were critical during loading and unloading with sinusal tachycardia episodes. It is necessary to extend the data set of animals to better understand the causes of these HR increases. Individual animal sensitivities and subjective evaluation of the events make the animals vulnerable to the stress (Locatelli 1989), making it difficult to distinguish between discomfort and stress and to estimate the threshold values of stress. As the animal's response is determined by complex interactions between genetic factors and experiences, it is impossible to generalize behaviors within the same or different breeds and species regarding various stressors. After the 20-h journey, the calves were not unloaded into the lairage cattle shed, but were transported for 2 additional hours to the destination farm. The handling of the animals during unloading and reloading in the lairage cattle shed causes not only further detrimental muscular strain but also an extension of the journey that leads to a marked change in homeostasis with a delayed return of biochemical blood parameters to normal (Bernardini et al. 2001). The stressors cannot be avoided, but as reported by Selye (1976), it is not necessary to completely remove the sources of stress, but instead to optimize them to make them tolerable and useful for survival and the execution of activities that the animals encounter. It is indispensable to execute a strategy that allows the farmers to work effectively within an international context and is characterized by increasing competition with new productive balances in light of national and community regulations.

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Chapter 11
The Association Between N-Methylglucamine Antimoniate and Pancreatitis in Dogs with Leishmaniasis

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Abstract In this study, the possible association between pancreatitis and treatment with methylglucamine antimoniate (MA) in dogs with leishmaniasis was evaluated. Of the 20 dogs included, three dogs showed clinical signs of pancreatitis. Abdominal ultrasound abnormalities suggestive of pancreatitis were observed in two dogs, and increased cPLI was observed in four cases. The statistical results showed a low probability of development of pancreatitis in patients treated with MA. However, considering the possible recurrence of pancreatitis in four cases in our study, we suggest that dogs treated with MA should be evaluated for pancreatitis, especially when compatible clinical signs are present.

Keywords Dog • Leishmaniasis • Meglumine antimoniate • Pancreatitis

Abbreviations

CanL	Canine leishmaniasis
cPLI	Canine pancreatic lipase immunoreactivity
MA	Meglumine antimoniate
UP/UC	Urinary protein/urinary creatinine ratio

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11.1 Introduction

N-methylglucamine antimoniate is the treatment of choice for canine leishmaniasis (CanL) (Mirò et al. 2008). In human medicine, there have been several cases of immunosuppressed patients with acute pancreatitis that was associated with meglumine antimoniate (MA) treatment of leishmaniasis (De Lalla et al. 1993; Barthet et al. 1994; Liorente et al. 2000; Kuyucu et al. 2001; Lambertucci et al. 2004). However, this relationship has been questioned by other authors (Shahian and Alborzi 2009). Veterinary data regarding this association are limited and the findings are not consistent (Aste et al. 2005; Xenoulis et al. 2010). The objective of this study was to further evaluate the possible association between pancreatitis and MA therapy in dogs affected by CanL.

11.2 Materials and Methods

In the period between January 2006 and December 2008, 77 dogs with suspected CanL were examined at the Department of Clinical Science of the University of Teramo. Of these, 33 dogs were shown to have CanL based on recently published guidelines (Paltrinieri et al. 2010). Of the 33 infected dogs, 13 dogs were excluded from the study because of other concurrent severe diseases that would have required treatment in addition to that for leishmaniasis (e.g. neoplastic, endocrine, hepatic, renal, gastrointestinal, and respiratory diseases). Twenty dogs were enrolled in the study. The following tests were performed for each dog: physical examination, hemato-biochemical profile, urinalysis, urine protein to creatinine ratio (UP/UC), and abdominal ultrasonography with particular attention on the evaluation of the pancreas.

The treatment of leishmaniasis consisted of subcutaneous administration of MA (Glucantime® Merial) at a dosage of 75 mg/kg every 12 h for a period of 60 consecutive days. Each dog was examined before treatment (T_0) and weekly for 9 consecutive weeks for a total of nine exams (T_0–T_9). At each examination, a clinical exam, a hemato-biochemical profile, a serum–protein electrophoresis, a urinalysis, and an abdominal ultrasound scan were obtained. Furthermore, serum specimens were collected and stored at −80°C until they were sent to a reference laboratory for pancreatic lipase immunoanalysis (Spec cPL™ Vet Med Lab IDEXX Laboratories, s.r.l.). For interpretation of Spec cPL values, results between 0 and 200 μg/L were considered normal, values greater than 400 μg/L were considered consistent with pancreatitis, and values between 201 and 399 μg/L were considered suspicious of pancreatic pathology.

Statistical analyses of the data were performed using *F*-tests (Variance Ratio Tests) for an estimation of the equality of the variance ratio of the values of Spec cPL among the different groups (T_0 and T_1–T_9). The Student's *t*-tests for parametrically matched data and Wilcoxon tests for non-parametrically-matched data were

used for comparisons between the mean values of cPLI at time T_0 with those at T_1–T_9. The odds-ratio method was used to verify the probability of an alteration in cPLI values in subjects being treated from T_0 to T_9. Statistical analyses were conducted using MedCalc Software Statistical® version 11.1 for Windows XP®. The value considered statistically significant was $P < 0.05$.

11.3 Results

The 20 dogs enrolled in this study were of different breeds (five mixed breeds, four Dobermanns, two English setters, and one each of Corso, Argeois, German shepherd, Boxer, Bloodhound, Rottweiler, Jack Russell, Pointer, Lagotto, and Kurzhar), different ages (1–8 years), both sexes (9 males and 11 females), and various weights (5.8–40 kg). None of the 20 dogs had a Spec cPL concentration greater than 400 mg/L or clinical signs suggestive of pancreatitis at T_0. During the subsequent weekly exams, 17 dogs showed no clinical signs suggestive of pancreatitis, two dogs had mild to moderate depression and anorexia, and one dog had vomiting and abdominal pain. In two of the three dogs with clinical signs, abdominal ultrasonography revealed abnormalities consistent with pancreatitis.

In subjects with suspected pancreatitis, administration of MA was suspended and a specific therapeutic treatment was undertaken. No significant hemato-biochemical alterations were found at T_0 or at subsequent time points (T_1–T_9). The physico-chemical tests of urine and urinary sediments were normal in all 20 dogs, while eight dogs had proteinuria with UC/UC ratios between 0.62 and 2.31 (average 1.36 ± 0.66 DS).

Comparisons of the mean values of the cPLI variance in subjects at T_0 and at subsequent time points did not show any statistically significant differences ($P > 0.05$). The odds-ratio analysis of the mean values of cPLI at T_0 and at subsequent time points suggested the absence of statistically significant differences at the various control points ($P > 0.05$).

11.4 Discussion

Pancreatitis in dogs can be described as acute or chronic, depending on the reversibility of histopathological changes (Steiner et al. 2008). The subclinical course of certain forms of pancreatitis, the nonspecific clinical signs in other cases, the operator- and instrument-dependent sensitivities, and the specificity of pancreatic ultrasonography all render definitive diagnoses difficult (Steiner 2003). The determination of pancreatic lipase by a commercially available immunoassay (Spec cPL) has recently been validated (Steiner 2003). This is an organ-specific and species-specific test that is both more sensitive (93%) and specific (78%) than

previously available laboratory tests for the diagnosis of pancreatitis (amylase, lipase, and TLI) (McCord et al. 2007).

Histological examination is the current definitive method of diagnosis, particularly in cases of chronic pancreatitis, but multiple biopsies are necessary since pathological lesions can be patchy in their distribution (Newman et al. 2004). However, pancreatic histology is difficult to perform in clinical practice because of practical considerations such as the critical condition of the patient and expenses.

In this study, the diagnosis of pancreatitis was made on the basis of compatible clinical and ultrasound signs and on the determination of Spec cPL values. The results from the comparison of the mean values of Spec cPLI in sick patients at T_0, at the beginning of treatment with MA, and at subsequent time points did not reveal any significant differences ($P > 0.05$). However, the analysis of single values showed an increase in Spec cPLI above the suggestive value for pancreatitis (>400 μg/L) in three out of 20 dogs at different times. In the first case, a cPLI value of 1,000 μg/L was detected at T_9 with clinical symptoms (mild depression and anorexia), and an ultrasound scan indicated pancreatic flogosis. In the second case, a value of 652 μg/L at T_5 was also associated with mild depression and anorexia, but there was no ultrasonographic evidence of pancreatitis. cPLI values of 493, 421, 584, and 468 μg/L were detected in an asymptomatic dog without ultrasonographic abnormalities on T_5, T_6, T_7, and T_8, respectively. A positive ultrasound scan for pancreatitis and compatible clinical signs (vomiting, abdominal pain, and/or depression) were associated with a cPLI value of 368 μg/L in one case at T_2. These data are in contrast with a recent survey where no clinical and biochemical evidence of pancreatitis was found in 23 dogs treated for leishmaniasis (14 with MA and nine with Allopurinol alone) (Xenoulis et al. 2010).

In our study, the lack of significant differences between the mean values of cPLI at T_0 and subsequent time points could be due to the particular pharmacokinetics of MA and the fact that the dosage used in this study (75 mg/kg subcutaneously every 12 h for 60 days) (Valladares et al. 1998) did not lead to a cumulative and progressive toxicity resulting in acute pancreatitis. The evaluation of serum-cPLI was made in retrospect, so the presence of symptoms and alterations during ultrasound scan was considered the priority during the weekly exams. Thus, in the case with clinically evident pancreatitis (clinical and ultrasound signs), treatment with MA was suspended, and MA treatments were not suspended in the case that lacked clinical and ultrasonography signs, but had Spec cPL values greater than the cutoff for pancreatitis at subsequent therapy time points (T_5–T_8). Mild symptoms were revealed in two subjects where the ultrasound signs were compatible with acute pancreatitis at T_9 and one dog was given specific treatments. Analysis of the odds-ratio values suggests that the probability that a dog being treated with MA for leishmaniasis will develop pancreatitis as compared to that of a dog with untreated leishmaniasis is not significantly greater.

The main limits of this study were the small number of subjects, the lack of a control group, and the impossibility of histologically diagnosing all dogs at all subsequent time points (T_0–T_9). In light of these limitations, it is the authors' opinion that pancreatitis is probably underestimated due to the difficulty of

diagnosis and nonspecific clinical findings. However, pancreatitis represents a complication in chronic inflammatory conditions like leishmaniasis. Further studies on pancreatitis as a complication in dogs with leishmaniasis treated with MA are warranted and underway. Despite our inability to definitively prove a link in this study, it is advisable that dogs being treated with MA should be evaluated for pancreatitis, especially when compatible clinical signs are present.

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Chapter 12
Superoxide Dismutase and Glutathione Peroxidase in Dogs with Leishmaniasis Following Antimoniate Therapy

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Abstract The effect of antimony treatment on antioxidative enzyme activities of erythrocyte superoxide dismutase (SOD) and blood glutathione peroxidase (GSHPx) and the associations between these enzyme activities and hematological indices of anemia (pre- and posttreatment) was studied in dogs with leishmaniasis. Twelve dogs with leishmaniasis before and after 60 days of therapy with *N*-methylglucamine antimoniate were used. No significant differences in antioxidant SOD and GSHPx activities or hematological indices, including HGB level, RBC number, HCT, MCV, MCH, and MCHC, were detected between pre- and post-antimony treatment. Results indicated that the presence or absence of previous treatment with antimony had no statistically identifiable effects on antioxidant enzyme activity or hematological indices.

Keywords Anemia • Canine visceral leishmaniasis • GSHPx • SOD • Therapy

Abbreviations

CanVL	Canine visceral leishmaniasis
GSHPx	Glutathione peroxidase
HCT	Hematocrit
HGB	Hemoglobin

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MCHC Mean corpuscular hemoglobin concentration
MCV Mean corpuscular volume
RBCs Red blood cells
ROS Reactive oxygen species
Sb^v Pentavalent antimony
SOD Superoxide dismutase

12.1 Introduction

Reactive oxygen species (ROS) contribute to the development and persistence of anemia during canine visceral leishmaniasis (CanVL) through multiple mechanisms (Weed and Reed 1966; Sen et al. 2001). The interaction between ROS and hemoglobin (HGB) results in the denaturation and precipitation of protein with the production of methemoglobin (Biswas et al. 1997), influencing the average lifespan of red blood cells (RBCs) (Weiss 1982). The peroxidation of RBC membrane lipids induced by ROS leads to the alteration of cell-membrane morphology and plasticity, promoting premature erythrocatheresis (Biswas et al. 1997). Among blood enzymes with free-radical scavenging activity, the erythrocyte form of superoxide dismutase (SOD) and the erythrocyte and plasmatic forms of glutathione peroxidase (GSHPx) are of particular interest (Guemouri et al. 1991). In our previous study, we detected a significant increase in the mean value of erythrocyte SOD activity and a significant reduction of RBCs and HGB in dogs with CanVL as compared with healthy dogs (Britti et al. 2008). In that study, the mean value of GSHPx activity in dogs with CanVL was decreased as compared with that of healthy dogs, even though this difference was not significant. The aim of this study was to evaluate the effect of Sb^v treatment on erythrocyte SOD and blood GSHPx activities in dogs with CanVL and to determine the potential associations between these enzyme activities and RBCs, HGB, and others indices of anemia before and after treatment.

12.2 Materials and Methods

Twelve dogs (7 males) of several breeds, average age 5.2 ± 2.0 years, with spontaneous CanVL as detected by serological (IFAT) and parasitological (lymph node cytology) methods, and with negative serological test results for ehrlichiosis and babesiosis, were enrolled in the study. Data were obtained at the time of diagnosis of CanVL (T_0) and after 60 days of therapy (T_{60}) with 75 mg/kg of *N*-methylglucamine antimoniate, SC q12h equivalent to 21.38 mg/kg of Sb^v. Twenty-five clinically healthy dogs (10 males) of several breeds, average age 4.1 ± 2.5, with no diseases as judged by the methods listed above, were used as controls (CTRL). From each of the 37 dogs, a 6.0-mL blood sample was drawn by

jugular venopuncture and divided into an EDTA tube for CBC analysis (Advia120®) and a LiHe tube for the measurement of antioxidative enzyme activities. Particularly, SOD activity was measured after isolation and lysis of RBCs, while GSHPx activity was determined in whole blood. Tests were performed using a clinical chemistry analyzer (Olympus-AU400®), according to the respective manufacturer's specification (Ransod® and Ransel®, RandoxLab).

Shapiro–Wilk tests indicated that the data followed a normal distribution. The Student's *t*-tests were used to evaluate the differences in the mean values of antioxidative SOD and GSHPx activities and hematological indices, including HGB, RBCs, HCT, MCV, MCH, and MCHC between T_0 and T_{60} groups (paired data) and between T_0 or T_{60} and CTRL groups (unpaired data). Linear regression analysis was used to evaluate the potential associations between these variables (pre- and posttreatment). All calculations were performed using a commercial statistical package (GraphPad Prism®).

12.3 Results

Means ± SDs, 95% confidence intervals ($CIs_{95\%}$) of the means, and *P* values for testing the differences between groups are reported in Tables 12.1 and 12.2. The comparison between T_0 and CTRL groups was reported in our previous study (Britti et al. 2008). In the current study, the comparison between T_0 and T_{60} groups revealed no significant differences in the mean values for SOD and GSHPx activities and the hematological indices, including HGB, RBCs, HCT, MCV, MCH, and MCHC levels. For example, no significant differences were detected between pre- and posttreatment with Sb^v for each of the considered variables in this study (Table 12.1). The comparison between T_{60} and CTRL groups revealed a mean level of SOD activity significantly higher ($P < 0.05$) at T_{60} and mean levels of HGB, RBCs, HCT, MCV, and MCH significantly lower ($P < 0.05$) at T_{60}. No significant differences were detected for GSHPx and MCHC levels (Table 12.2). Simple linear regression analyses demonstrated significant negative associations among the variables at T_{60}, similar to those detected at T_0 in our previous study

Table 12.1 Hematological indices and activities of antioxidative enzymes in dogs with CanVL before (T_0) and after (T_{60}) Sb^v therapy

	T_0 Mean ± SD ($C.I._{95\%}$)	T_{60} Mean ± SD ($C.I._{95\%}$)	$P < 0.05$
RBC (x106/μL)	5.64 ± 0.83 (5.11–6.17)	5.49 ± 0.8 (4.98–6.0)	n.s.
HGB (g/dL)	12.42 ± 2.04 (11.12–13.71)	12.46 ± 2.0 (11.19–13.73)	n.s.
HCT (%)	35.67 ± 6.01 (31.86–39.49)	35.43 ± 5.41 (32.0–38.87)	n.s.
MCV (fL)	60.99 ± 9.2 (55.15–66.84)	64.58 ± 3.4 (62.42–66.75)	n.s.
MCH (pg)	21.69 ± 2.24 (20.27–23.11)	22.72 ± 1.8 (21.58–23.86)	n.s.
MCHC (g/dL)	33.78 ± 4.44 (30.95–36.6)	35.15 ± 1.57 (34.15–36.15)	n.s.
SOD (U/gHGB)	114.2 ± 18.0 (102.7–125.6)	115.2 ± 21.6 (101.5–129.0)	n.s.
GSHPx (U/L)	988.7 ± 222.3 (847.5–1130.0)	872.1 ± 271.9 (699.3–1044.8)	n.s.

Table 12.2 Hematological indices and activities of antioxidative enzymes in dogs with CanVL after Sb^v therapy (T_{60}) and in healthy dogs (CTRL)

	T_{60} Mean ± SD (C.I.$_{95\%}$)	CTRL Mean ± SD (C.I.$_{95\%}$)	$P < 0.05$
RBC (x106/μL)	5.49 ± 0.8 (4.98–6.0)	6.94 ± 0.61 (6.68–7.2)	<0.0001
HGB (g/dL)	12.46 ± 2.0 (11.19–13.73)	16.86 ± 1.67 (16.16–17.57)	<0.0001
HCT (%)	35.43 ± 5.41 (32.0–38.87)	47.65 ± 4.55 (45.72–49.57)	<0.0001
MCV (fL)	64.58 ± 3.4 (62.42–66.75)	68.58 ± 2.78 (67.41–69.76)	<0.001
MCH (pg)	22.72 ± 1.8 (21.58–23.86)	24.3 ± 1.28 (23.76–24.84)	<0.01
MCHC (g/dL)	35.15 ± 1.57 (34.15–36.15)	35.43 ± 2.22 (34.49–36.37)	n.s.
SOD (U/gHGB)	115.2 ± 21.6 (101.5–129.0)	82.6 ± 9.0 (78.8–86.4)	<0.0001
GSHPx (U/L)	872.1 ± 271.9 (699.3–1044.8)	1025.3 ± 284.6 (905.2–1145.5)	n.s.

(Britti et al. 2008). Particularly, simple linear regression analysis indicated a strong negative linear associations between SOD activity and HGB concentration ($R^2 = 0.96$; $P < 0.0001$), SOD activity and RBCs ($R^2 = 0.76$; $P < 0.0001$), and SOD activity and HCT ($R^2 = 0.90$; $P < 0.0001$) at T_{60}. No significant associations were detected at T_{60} between SOD and GSHPx activities and between GSHPx activities and hematological indices. Additionally, a minimal negative association ($R^2 = 0.38$; $P < 0.05$) was detected between SOD activities at T_0 and HGB concentrations at T_{60}, and a minimal positive association ($R^2 = 0.39$; $P < 0.05$) was detected between GSHPx activities at T_0 and HGB concentrations at T_{60}. Finally, a multiple linear regression analysis was used to develop a moderately accurate algorithm ($R^2 = 0.50$; $P < 0.05$) to predict HGB concentrations at T_{60} by measuring SOD and GSHPx activities at T_0. The relationship between these variables (in the absence of colinearity between SOD and GSHPx at T_0) is described by the following formula: HGB_{T60} (g/dL) = 13.901 + 0.004 × $GSHPx_{T0}$ (U/L) − 0.045 × SOD_{T0} (U/gHGB). In the developed algorithm, parameters of regression indicate a positive association between $GSHPx_{T0}$ activities and HGB_{T60} concentrations and a negative association between SOD_{T0} activities and HGB_{T60} concentrations.

12.4 Discussion

Results of our study revealed that the presence or absence of a previous treatment with Sb^v had no statistically identifiable effect on antioxidative enzyme activities and hematological indices. No significant differences in SOD and GSHPx activities and hematological indices were detected in dogs with CanVL before and after therapy with Sb^v (T_0 and T_{60}). Erythrocyte SOD activities were significantly higher and HGB, RBCs, HCT, MCV, and MCH levels were significantly lower in dogs with CanVL at 60 days after therapy as compared to clinically healthy dogs (Figs. 12.1 and 12.2). On the basis of these findings, we assume that these parameters are significantly altered in dogs with CanVL as compared with those of control dogs, regardless of the Sb^v treatment. This result suggests that in dogs

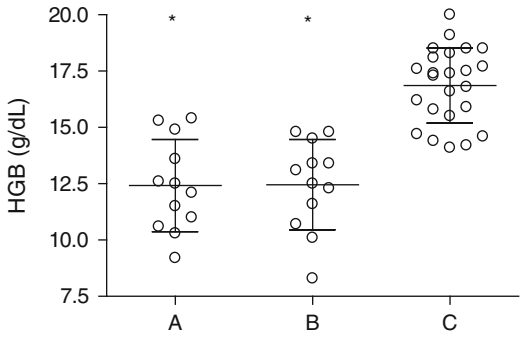

Fig. 12.1 *Dots* represent individual HGB (g/dL) values. *Horizontal lines* represent mean ± SD. A = T_0; B = T_{60}; C = CTRL. *$P < 0.05$ as compared to CTRL

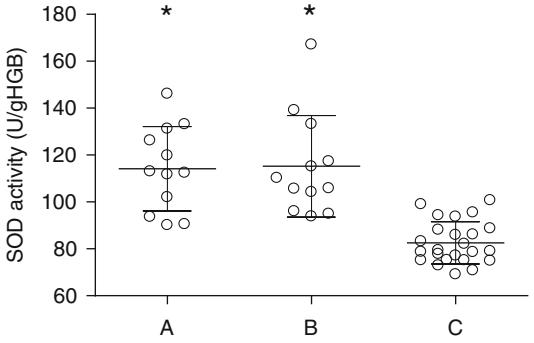

Fig. 12.2 *Dots* represent individual erythrocyte SOD (U/gHGB) values. *Horizontal lines* represent mean ± SD. A = T_0; B = T_{60}; C = CTRL. *$P < 0.05$ as compared to CTRL

with CanVL, Sb^v therapy does not improve the redox state or the underlying anemia. This may be explained, at least in part, by other antioxidative systems not evaluated in this study, by the degree of renal impairment or failure, which is not always detectable through routine tests (Ciaramella et al. 1997), or by the fact that the Sb^v therapy alone may not be sufficient to determine the improvement of the hematological indices and redox states in dogs with CanVL (Erel et al. 1999). For example, in hamsters with experimental visceral leishmaniasis, the combination of antioxidants, such as α-tocopherol, ascorbic acid, and flavonoids, with the Sb^v therapy resulted in a greater reduction of ROS and parasitemia (Sen et al. 2005), and a greater increase in HGB concentrations and RBC half-lives as compared to the Sb^v therapy alone (Sen et al. 2004). It is important to note that while the levels of SOD activity and hematological indices in dogs with CanVL were similar before and after therapy with Sb^v, but significantly different as compared to those of control dogs, the levels of GSHPx activities before therapy were similar to that of control dogs, and were basically reduced after Sb^v therapy (Fig. 12.3). As previously reported during chronic experimental infections in hamsters (Sen et al. 2001) and cutaneous leishmaniasis in humans (Kocyigit et al. 2003), this finding, although without statistical significance, may suggest a depletion of the GSHPx antioxidative system during chronic CanVL infections in dogs. However, the lack of a statistical significance for this finding underlines the need for increased sample size.

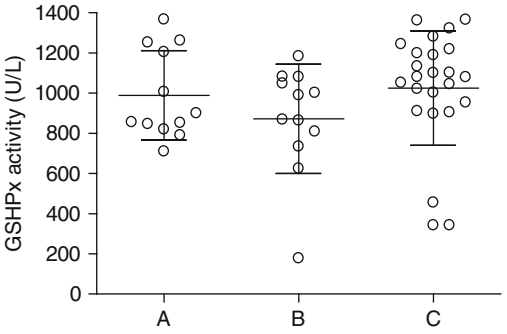

Fig. 12.3 *Dots* represent individual blood GSHPx (U/L) values. *Horizontal lines* represent mean±SD. A = T_0; B = T_{60}; C = CTRL

Interestingly, pretreatment GSHPx activity is positively related with the progression of anemia, while pretreatment SOD activity is inversely related. Although significant, the coefficient of determination R^2 of the developed algorithm indicates that these relationships are weak. Thus, despite a 50% variance in posttreatment HGB concentrations, this may be explained by the variance of pretreatment SOD and GSHPx activities, and the remaining percentage is due to other factors, such as low iron concentrations, renal or bone marrow impairment, or other antioxidative systems not considered here. Nevertheless, the normal distribution of the data and the absence of colinearity between SOD and GSHPx activities support the evaluation of different pretreatment antioxidative systems that may be predictive or protective against the oxidative damage involved in anemia during CanVL. To the authors' knowledge, this is the first study on the comparison of SOD and GSHPx activities in dogs with CanVL before and after therapy with Sb^v. As previously observed during CanVL (Bildik et al. 2004), RBCs are exposed to an oxidative stress that could be responsible for their premature removal from circulation. Our results suggest that this damage persists even after Sb^v therapy. A study is in progress to examine the efficacy of the combination of antioxidants with the Sb^v therapy in dogs with CanVL.

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Chapter 13
The Importance of the New Institution of "Mediation" for the Civil Liability of Veterinarians

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Abstract Veterinarians, by the nature of their occupation, deal with animals on a daily basis and in a variety of contexts. The core of their activities involves the provision of professional services, which are usually performed to the satisfaction of both the animal and the owner. Not all of the interactions have the desired outcome, however, and veterinarians who have been defendants in lawsuits find themselves confronted with a wide variety of legal claims. Lawsuits that involve the assessment of medical liability generally take a long time, and in recent years, the number of court actions against veterinarians has increased. In view of this fact, we examine legislative decree no. 28/2010, which also concerns civil proceedings on liability in human medicine. We forecast that the new institution of "mediation" could become an important instrument in hastening the resolution of court actions concerning medical liability.

Keywords Civil law • Mediation • Professional liability • Veterinarian

13.1 Introduction

The veterinary, medical, and dental professions are defined in the Italian Civil Code (c.c.) as "*intellectual*" (art. 2229 c.c.). When a veterinarian accepts the task of treating an animal, he/she puts him/herself under an obligation to perform a specific job for the owner, meaning that both the veterinarian and client (in this case, the owner) stipulate a contract called "contract of intellectual work," which involves rights and mutual obligations (Panichi et al. 2002, 2003).

It should be emphasized that the obligations under which a veterinarian works have special connotations because of the sensitive situations in which he/she often

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has to operate and the type of liability that may arise. First, it should be noted that the provision of medical care in general, veterinary care in this case, involves obligations that belong to the category of "means" and not "results."

The veterinarian has no obligation under law to guarantee concrete results from his/her professional services. However, he/she must do whatever is technically possible to progress toward the result, and ideally even attain it, with the consequence that his/her possible failure – for example the death of the animal – should be evaluated only along the lines of duties of professional activities and, in particular, in relation to the "duty of care." The duty of care should be respected in any type of contract, as required by the first paragraph of art. 1176 c.c. that says, "*In carrying out his obligation, the debtor must use the diligence of a good father* . . ." but, concerning the obligations inherent in professional activity (including medical activity), the second paragraph continues by saying, ". . . *the diligence must be assessed with regard to the nature of the business*."

Given the foregoing and given the nature of a medical professional's obligation as one of methods and not one of results, it follows that, for veterinarians, each case should be assessed on its own merits, as far as actual diligence and practical results are concerned. This is specifically the degree of diligence that should be used while carrying out professional activities (e.g., examination, diagnosis, surgical procedures, and prescription of drugs) and the levels of professional capacity and attention (Panichi et al. 2002). However, in cases where the professional services to be performed on animals involve solving particularly difficult and complex technical problems – for example, complex surgery that is still at an experimental stage – professional responsibility for a lack of success is less marked and exists only in cases of serious misconduct, willful, or gross negligence, that is, behavior that is medical malpractice (Passantino 2002).

13.2 Legal Aspects

The above reflections aim to introduce the procedural aspects of the veterinarian's responsibilities in this delicate and topical matter.

The most complicated aspect of lawsuits relating to the establishment of medical liability is essentially one concerning the "proof" of responsibility itself. Most frequently, in fact, the judicial authority's knowledge and skills are insufficient to enable it to understand to what degree of care the doctor should have used and, accordingly, whether an injury worthy of compensation is a consequence of the doctor's conduct.

In fact, when a veterinarian is charged with a liability, whether civil or criminal, the party who claims to have suffered damages must, on the one hand, prove, ideally with the help of technical advice, negligence in the performance of professional services, as well as the existence of damage and the causal relationship between the defective or inadequate job performance and the damage deriving therefrom; on the other hand, as the judicial organ likely lacks the technical

knowledge necessary to judge both the level of care required by the veterinarian in the specific case and the existence of a causal link between the harmful action and the event, it must necessarily avail itself of a technical consultant or expert so that an expert opinion may solve doubts and fill gaps in the judge's knowledge.

The burden of proof that the efforts in the execution of professional services were adequate to the case lies with the professional concerned, however. Therefore, in lawsuits relating to claims for medical liability damages, both in human and in veterinary medicine, it is clear that medico-legal technical consultancy has a key role in determining whether the event with which the veterinarian is charged (i.e., death, worsening of the disease) is actually due to professional misconduct or to other factors that will exonerate the veterinarian from professional responsibility.

13.3 Discussion

The foregoing brief remarks, in substance, show how and why legal proceedings relating to the investigation of medical liability, of whatever kind, take a very long time to reach a conclusion, because of their complexity and the way in which this particular responsibility must be established and verified in practice. Moreover, in recent years, lawsuits against veterinarians have increased exponentially, with a consequent overloading of the judicial system. Legislative Decree No. 28/2010, which introduces a new procedural method to be activated in the field of actions for damages resulting from medical liability, is to be welcomed, as it should help speed up the settlements of such legal disputes (Anon 2010). It establishes the institution of mediation aimed at reconciliation, which is an obligatory preliminary stage for those who wish to take legal action to assert their right to compensatory damages against a doctor/veterinarian considered responsible for those damages through professional conduct.

Specifically, the decree defines *"mediation"* as "*the activity carried out by an impartial third party aimed at assisting two or more subjects, both in the search for an amicable agreement for the settlement of a dispute and in the formulation of a proposal for the solution of the same."* The term *"conciliation"* means, however, *"the settlement of the dispute after mediation has been carried out,"* that is, a solution achieved through mediation, while the term "*Organ of conciliation"* indicates the *"public or private body where the mediation process may take place."*

It is important to fully understand what the legislators were aiming to achieve with the introduction of this obligatory stage before moving on to the usual legal procedures to make an analysis of possible practical consequences for the health professions. Analysis of the principles behind the enabling act whose direction the government must follow when emanating the related law shows clearly that the legislator's intention was to create an alternative system that would be more agile and speedy than ordinary procedures. Indeed, it is now mandatory for specific civil and commercial proceedings and is available in all cases for disputes concerning *available rights* (that is to say, those rights for which the holder may act in terms of

availability, of waiver of transfer, thus property rights in general). This method of dispute settlement is indubitably of great significance because, even though it is obligatory, it is not a substitute for the ordinary systems of justice. In other words, although this procedural stage is obligatory, it must in no way *"preclude access to justice."* Indeed, even when the professional bodies that are in charge of it have a requirement of independence (for example, judges) and offer conciliation/mediation services in the long term, they do not have the faculty to pronounce any decisional sentence (which arbitration bodies may do). The only pronouncement made by "*mediators"* that can produce juridical effects between the parties is the "*Statement of Agreement*" that, once it has been examined by the presiding judge in whose district the professional body is and has been found to be both formally and substantially in order, may become an actual sentence by means of a decree of homologation. It is of interest here to see what bodies may aspire to become an "Organ of conciliation." Article 16 of the Legislative Decree specifies that only those public and private bodies that can guarantee efficiency and reliability will be permitted to intervene in mediation; inclusion in a special register is also necessary.

It should also be noted that the councils of professional associations might, after receiving authorization from the Ministry of Justice, establish special bodies from their own staff and on their own premises to deal with matters relating to their specific spheres of expertise. Another important innovation, which can also have a useful deterrent function, is the provision of special rules concerning the payment of court costs. In contrast to the rule that has the loser of the case pays the costs, the regulations examined here contemplate the possibility that, in those cases where the sentence pronounced in the ordinary proceedings exactly corresponds to the agreement set out in the conciliation phase, the judge may decide not to recoup the costs incurred by the winner if the latter rejected the agreement at the earlier stage and, moreover, sentence him/her to repay the loser's costs and a further sum as a contribution to court expenses. The possibility of being sentenced to pay damages remains, if the judge decides that the party has undertaken a "reckless" lawsuit, as well as a subsequent sentence to pay the fees of experts who may have assisted the organs of conciliation in lawsuits where specific technical knowledge is required.

In the light of the innovations in civil proceedings relating to medical liability disputes, the new institution of mediation should be welcomed as, in the future, it could become a valuable instrument for the settlement of disputes. By taking a constructive approach, mediation allows the parties involved to focus on the real interests at stake and will, if time scales are evaluated appropriately, enable favorable agreements for all parties to be reached without long delays. Above all, this new institution will make it possible to avoid high legal costs, which are always incurred when experts' reports or court-appointed technical consultancies are involved. Now, therefore, it seems possible that doctors in general and veterinarians in particular will have to face a significantly smaller number of court cases than in the past also because veterinarians often face court action as a consequence of the owner's pique or resentment at the loss of an animal, rather than as a result of actual, tangible negligence on their part.

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Chapter 14
Increased Oxidative/Nitrosative Stress in Bitches with Several Tumors

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Abstract Experimental and clinical data suggest that reactive oxygen species, which cause no damage under physiological conditions, can cause cellular damage at high concentrations and may play key roles in carcinogenesis. The aim of this study was to evaluate the activities of catalase, superoxide dismutase, and glutathione peroxidise, as well as the production of malondialdheyde and nitric oxide, in bitches suffering adenocarcinoma, histiocytoma, and hemangiopericytoma. Data showed that the levels of both malondialdheyde and nitric oxide were significantly increased in both plasma and tissues. The decreased activities of antioxidant enzymes and increased oxidative stress observed could be attributed to a reduction in antioxidant defenses of the organism because of depleted defenses over time.

Keywords Catalase • Glutathione peroxidase • Superoxide dismutase • Tumor

Abbreviations

CAT	Catalase
GPx	Glutathione peroxidase
MDA	Malondialdehyde

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ROS Reactive oxygen species
SOD Superoxide dismutase

14.1 Introduction

Reactive oxygen species (ROS) and nitrogen species together with superoxide and hydroxyl radicals and hydrogen peroxide play key roles in carcinogenesis (Borek 1983). Their toxicity is based on the ability to induce lipid peroxidation, resulting in damage to cell membranes, proteins, polysaccharides, and nucleic acids. It has been shown that the production of ROS is increased in tumor cells, leading to oxidative stress responsible for DNA damage and genomic instability and possibly favoring tumor progression (Suh et al. 1999). In addition, during tumor progression, high levels of ROS are responsible for the activation of transcription factors, such as nuclear factor (NF)-κB activator protein I (Arnold et al. 2001). Our recent research has shown, in lymphocytes from patients with chronic myeloid leukemia, calcium channel hypofunction due to cellular oxidative stress (Ciarcia et al. 2010). The objective of this study was to evaluate the expression of markers of oxidative stress in dogs suffering from breast adenocarcinoma, hemangiopericytoma, and histiocytoma.

14.2 Materials and Methods

Thirty dogs of different breeds, aged between 6 and 13 years, including eight patients with breast adenocarcinoma, six with histiocytoma, five with hemangiopericytoma, and 11 controls were used in this study. The diagnostic findings were provided by the Surgical Clinic of our Faculty. Blood samples were collected in heparinized tubes. The plasma obtained was used for thiobarbituric acid, nitrite, and nitrate assays and for CAT, SOD, and GPx assays. Bitches, before surgery, were premedicated with atropine sulfate at a dose of 0.05 mg/kg administered subcutaneously with a combination of 20 μg/kg medetomidine and 0.2 mg/kg acepromazine administered intramuscularly. Anesthesia was performed with propofol injected at a dose of 7 mg/kg intravenously (bolus) and maintained with isoflurane in O_2. At the end of surgery, a portion of tumor tissue was removed and stored at −80°C. The samples were then thawed and homogenized in a solution of 50 mM potassium phosphate and 0.1% Triton (pH 7.0). The homogenates were centrifuged at 15,300 rpm for 30 min at 4°C, and the supernatant was used for determining the activities of antioxidant enzymes and analytes. The CAT assay was performed using a colorimetric method according to Ding et al. (2000), the SOD assay using a colorimetric method according to Sun et al. (1988), and the GPx assay using a colorimetric method according to Mannervik et al. (1985). Lipid peroxidation was determined by the measurement of MDA through the thiobarbituric acid

test according to Armstrong et al. (1998). Nitrates and nitrites were assayed by means of the Griess reagent (Moncada 1992). Plasma proteins were determined by the method according to Lowry et al. (1951).

14.3 Results

Table 14.1 shows the activities of antioxidant enzymes. The activities of SOD and CAT were significantly reduced in adenocarcinoma (43.4% and 74.5%), in hemangiopericytoma (−23.6% and −48.0%), and in histiocytoma (14.7% and 17.3%). In contrast, GPx activity was significantly increased in adenocarcinoma (251.5%) and in hemangiopericytoma (16.3%) but was unchanged in histiocytoma. Table 14.2 shows the results of the MDA production and nitrite assays. In patients with adenocarcinoma, the levels of MDA and nitrite significantly increased with respect to control from 101.1 ± 11.5 to 152.7 ± 18.4 pmol/mg protein and from 34.5 ± 3.9 to 56.4 ± 6.1 in plasma, and from 107.8 ± 9.7 to 148.4 ± 15.7 pmol/mg protein and 40.7 ± 4.4 to 86.5 ± 9.9 in the tissue, respectively. In subjects with hemangiopericytoma, the MDA and nitrite levels significantly increased with respect to control from 97.7 ± 9.9 to 131.9 ± 14.8 and from 31.9 ± 3.6 to 44.8 ± 5.3 in plasma and from 109.7 ± 13.7 to 127.2 ± 13.9 and from 44.9 ± 5.3 to 105.2 ± 2.8 pmol/mg protein in the tissue, respectively. No significant changes were observed in patients with histiocytoma in terms of MDA and nitrite production.

Table 14.1 Antioxidant enzyme activity in cytosol of various tissues (mean ± SD)

Samples	SOD % inhibition	GPx U/ml Δ%		CAT μmoles/min/ml Δ%	
Control skin (6)	10.6 ± 2.5	0.104 ± 0.04	–	491.1 ± 51.7	–
Histiocytoma (6)	17.3 ± 2.1	0.107 ± 0.03	2.9	418.5 ± 29.8	−14.7
Hemangiopericytoma(5)	*23.6 ± 2.7	*0.121 ± 0.04	16.3	*255.3 ± 26.3	−48.0
Control breast (5)	10.1 ± 2.1	0.097 ± 0.05	–	510.2 ± 47.9	–
Adenocarcinoma (8)	*43.4 ± 4.2	*0.341 ± 0.08	251.5	*129.8 ± 15.3	−74.5

*$P < 0.01$ compared with controls

Table 14.2 Nitrite and MDA levels in plasma and cytosol of various tissues (mean ± SD)

Samples	MDA (pmol/mg protein)		$NO_2 + NO_3$ (pmol/mg protein)	
	Plasma	Cytosol	Plasma	Cytosol
Control skin (6)	97.7 ± 9.9	109.7 ± 13.7	31.9 ± 3.6	44.9 ± 5.3
Histiocytoma (6)	106.4 ± 9.3	104.4 ± 12.6	26.5 ± 5.6	59 ± 6.6
Hemangiopericytoma (5)	*131.9 ± 14.8	*127.2 ±13.9	**44.8 ± 5.3	**105.2 ± 12.8
Control breast (5)	101.1 ± 11.5	107.8 ± 9.7	34.5 ± 3.9	40.7 ± 4.4
Adenocarcinoma (8)	**152.7 ± 18.4	** 148.4 ± 15.7	**56.4 ± 6.1	**86.5 ± 9.9

*$P < 0.05$ compared with controls, ** $P < 0.01$ compared with controls

14.4 Discussion

Experimental and clinical data suggest that high concentrations of ROS can cause nitration and/or oxidation of cellular proteins with alterations in several transcription factors that play key roles in both carcinogenesis and drug-induced toxicity (Portakal et al. 2000). SOD, GPx, and CAT are considered the main antioxidant enzymes, being directly involved in the elimination of ROS produced during normal cellular metabolism or after oxidative insult. The activities of these enzymes can vary depending on the phenotype of the tumor, being increased in some and decreased in others. Jung et al. (1997) reported decreased or unchanged activities of these enzymes in prostate cancer. In contrast, Baker et al. (1997) detected lower levels of antioxidant enzymes in malignant prostate epithelial cells compared to benign prostate epithelial cells. The production of oxygen radicals increases with tumor progression, implying greater lipid peroxidation, which induces the degeneration of cell membranes and DNA damage. In our study, levels of MDA and nitrite in dogs suffering from adenocarcinoma and hemangiopericytoma were significantly increased in both plasma and tissue. It has been demonstrated that superoxide anion and hydroxyl radicals may inactivate the antioxidant enzymes, particularly catalase, reducing cellular defenses against free radicals (Mayo et al. 2003). The decreased activities of antioxidant enzymes and increased oxidative stress observed in our study agree with the results described by Pejić et al. (2009) in endometrial adenocarcinoma and may be attributed to a reduction in antioxidant defenses of the organism. In conclusion, our results confirm an alteration in oxidative stress in tumor cells, but further studies are needed to understand whether the observed changes in patients with malignant tumors are the cause or the consequence of increased oxidative stress.

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Chapter 15
Validation of Thromboelastometry in Cattle

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Abstract Thromboelastometry (TEM) and thromboelastography are useful tools for the evaluation of the entire clotting process from the activation of plasmatic factors until fibrinolysis. TEM has been validated in several domestic species but not in cattle. In this study, we defined normal TEM values in adult cattle and in veal calves; we then compared rates of coagulation in these two groups. Furthermore, the precision of the instruments was evaluated. Finally, we studied the correlation between the hematological and TEM results. In calves, we identified a faster activation of plasmatic factors (shorter clotting times) and lower fibrinogenemia. The precision of TEM on the bovine blood samples was good. Additionally, hematocrit was positively linked to clotting time of the intrinsic and extrinsic pathways, whereas fibrinogenemia was strongly associated with fib-TEM® tracing.

Keywords Bovine • Coagulation • Milk-fed veal calves • Reference range • Thromboelastometer

15.1 Introduction

There exists a wide range of veterinary laboratory tests for blood coagulation evaluation, each analyzing a specific phase of the coagulation cascade: primary hemostasis, secondary hemostasis, and fibrinolysis. Unlike other tests, the thromboelastometer (TEM)/thromboelastograph assesses the blood's viscoelastic properties during all phases of clot formation. Additionally, through the addition of specific reagents, the TEM allows for the selective study of the intrinsic pathway (in-TEM®), the extrinsic pathway (ex-TEM®), and fibrinogen function (fib-TEM®) (Luddington 2005).

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Most coagulation studies in bovine species have investigated, using direct or indirect methods, plasmatic factor activity. For example, some have described hemostasis in specific physiologic conditions such as pregnancy or peripartum (Heuwieser et al. 1990) or within the first month of life of the calf (Gentry et al. 1994). Among the most common coagulation disorders are hypocoagulability conditions that may be congenital, for example, plasma factor deficiency, or acquired, for example, toxic, carential, or immune-mediated (Radostis 2007). In addition, disseminated intravascular coagulation has been associated with abomasal displacement (Sobiech et al. 2008), septic shock (Irmak et al. 2006), and traumatic reticuloperitonitis (Gokce et al. 2007). Reports on hypercoagulability conditions are scarce. Hypercoagulability is a sequela of acquired systemic disease (Pusterla et al. 1997; D'Angelo et al. 2006), and a congenital predisposition to thrombophilia is unknown in cattle (Gentry 2004). Recently, a hypercoagulable state following steroidal drug administration in calves was detected by TEM (Borrelli et al. 2009). The use of TEM, also in cattle, could be helpful for the simultaneous evaluation of hemostatic components, early detection of hypercoagulability, and hyperfibrinolysis, all of which may be difficult to diagnose with conventional tests.

The aim of this study was to evaluate the analytical performance of TEM in adult cattle and veal calves and to define normal reference ranges in these animals.

15.2 Materials and Methods

The study population was 62 animals: 48 adult cattle (mean age, 23 months; range, 11–135 months) and 14 Holstein–Friesian veal calves (all males; age, 6 months). The adult animals were 15 Piedmontese females and 33 males of different breeds (five Charolais, two Piedmontese, and 26 Holstein–Friesian). All animals were judged healthy according to physical examination, complete blood count (CBC), and blood biochemistry. Cattle that had received pharmacological treatment within the month before the beginning of the study or were noted to have spontaneous bleeding, in the final month of pregnancy, or in the first month after calving were excluded. Blood samples were drawn from the jugular vein with 20-gauge needles and collected separately into EDTA-containing tubes for CBC (ADVIA 120 Hematology System, Siemens Diagnostics), 3.8% sodium citrate tubes for TEM (ROTEM®, TEM Innovations GMBH) and coagulation profile (coagulometer STart®, Diagnotica Stago), and tubes with clot activator for blood biochemistry (Ilab300plus, Instrumentation Laboratory).

Thromboelastometric analyses were carried out in the field within 15 min after blood collection, and the in-TEM®, ex-TEM®, fib-TEM®, and na-TEM® profiles were performed according to the manufacturer's instructions. The following parameters were evaluated for each coagulation profile: clotting time [(CT), s], clot

formation time [(CFT), s], maximum clot firmness [(MCF), mm], and α angle (°). In addition, the maximum velocity [(V_{max}), mm/min] and area under the curve [(AUC), mm^2] were evaluated in the na-TEM® profile. A standard coagulation profile, including prothrombin time [(PT), s], activated partial thromboplastin time [(aPTT), s], and fibrinogen (mg/dl), was also performed in 26 animals (16 adults and 10 calves).

Hematological and thromboelastometric values are expressed as the mean ± standard deviation (SD), 5th–95th percentile, and median. Data distribution was tested with the Shapiro–Wilk test. The coefficients of variation (CV) were calculated on duplicate tracings performed simultaneously for the imprecision test. The Wilcoxon test was used to compare hematological and thromboelastometric results for adults and calves. Correlations between TEM and hematologic variables [erythrocyte count (RBC), hematocrit (HCT), PT, aPTT, and fibrinogen] were investigated using the Spearman test. Statistical significance was set at the less than or equal to 0.05 probability level. Statistical analysis was performed using the open source software R 2.7.0 (R Development Core Team 2008).

15.3 Results

CBC and blood biochemistry were within normal limits for adult cattle. Microcytic hypochromic anemia was observed in the calves (HCT 20.6 ± 3.6%; MCV 24.5 ± 3.3 fL; hemoglobin 8 ± 1.4 g/dL); HCT was significantly lower in calves ($p < 0.001$) than in adults. The standard coagulation profile showed a significant decrease in PT and a significant increase in aPTT and fibrinogen in adults versus calves: PT 23.5 ± 2.9 s versus 25.7 ± 1.6 s; aPTT 36.8 ± 9.6 s versus 27.4 ± 3.6 s; fibrinogen 390.9 ± 125.4 mg/dl versus 259.9 ± 43.6 mg/dl, respectively.

The TEM reference values for adults and calves are reported in Tables 15.1 and 15.2, respectively. In the calves, the CT was significantly shorter for the in-TEM® ($p = 0.008$), ex-TEM® ($p < 0.001$), and fib-TEM® ($p = 0.006$) profiles, whereas the CFT was significantly longer ($p = 0.03$) for the fib-TEM® profile.

Concerning instrument precision, a lower CV was noted for MCF (range from 0.9% for in-TEM® to 8.5% for fib-TEM®), for α angle (range from 2.4% for in-TEM® to 12.5% for na-TEM®), and for AUC (3.1%). Higher CVs were observed for CT (range from 6.3% for ex-TEM® to 12% for na-TEM®), for CFT (range from 5.4% for ex-TEM® to 15.7% for na-TEM®), and for V_{max} (25.7%).

A positive correlation was found between HCT and CT both for in-TEM® ($r = 0.4$; $p = 0.006$) and ex-TEM® ($r = 0.5$; $p < 0.001$) and between HCT and aPTT ($r = 0.6$; $p < 0.001$). For the fib-TEM® profile, fibrinogen correlated positively to CT ($r = 0.4$; $p = 0.02$), MCF ($r = 0.35$; $p = 0.03$), and α ($r = 0.4$; $p = 0.006$), and negatively to CFT ($r = -0.5$; $p < 0.001$).

Table 15.1 Thromboelastometer results for adult cattle

Test	CT (s)	CFT (s)	MCF (mm)	Alpha (°)	V_{max} (mm/min)	AUC (mm^2)
In-TEM	353.3 ± 108 (354) 219.5–565.7 (*)	83.2 ± 67.3 (67.5) 42–107.4	79 ± 4.9 (79.5) 71–86.7	74.7 ± 8.1 (76.5) 65.5–81	na	na
Ex-TEM	87.9 ± 18.8 (86) 64.9–113.3 (*)	98.7 ± 36.6 (98) 46.3–154.3	78.5 ± 4.2 (79) 72–85.4	74.7 ± 4.9 (75) 67.3–81.7	na	na
Fib-TEM	82.2 ± 16.7 (81) 60.2–104.8 (*)	121 ± 78.8 (103) 45.2–219.7 (*)	33.2 ± 6.2 (32) 24.3–44	75 ± 5.2 (76) 66.6–81.7	na	na
Na-TEM	1565 ± 581.2 (1,534) 725.5–2643.5	549 ± 281.6 (511) 234.7–967.9	64.7 ± 16.9 (71.5) 29.1–82.7	35.7 ± 12.5 (34) 17.2–54.7	8.17 ± 5.2 (7) 4–20	7,190 ± 1,697 (6498) 2,990–8,277

Values are expressed as mean ± SD (median), and 5th–95th percentile; *na* not applicable; * indicates significant differences between adults and calves

Table 15.2 Thromboelastometer results for calves

Test	CT (s)	CFT (s)	MCF (mm)	Alfa (°)
In-TEM	270.6 ± 66.1 (274.5) 173.5–360.3 (*)	64.9 ± 13 (68) 45.9–81	74.9 ± 10.3 (77) 62.1–81	77.2 ± 2.4 (76.5) 74.6–81
Ex-TEM	70.3 ± 9.9 (71) 55.7–81.7 (*)	99.8 ± 19.3 (103.5) 68.2–122.1	77.7 ± 3 (78) 72.9–81.3	74.1 ± 3.1 (74.5) 70–78
Fib-TEM	73.9 ± 6 (74.5) 65.6–81.7 (*)	183.1 ± 105 (152.5) 66.6–374.9 (*)	30.1 ± 4.1 (30.5) 24.6–36	75.9 ± 3.5 (76.5) 69.9–79.7

Values are expressed as mean ± SD (median), and 5th–95th percentile; * indicates significant differences between adults and calves

15.4 Discussion

Compared to equine (Paltrinieri et al. 2008) and canine (Wiinberg et al. 2005; Lubas et al. 2006) tracings, bovine thromboelastograms present several distinctive features. These differences are due to higher platelet counts and fibrinogen levels in cattle (Kramer 2006). CFT, which depends on initial platelet and fibrinogen activation, is short in cattle; MCF, which expresses maximum clot consistency and depends on the interaction between platelets, fibrinogen, and factor XIII, is increased in cattle. In our study, CT, which represents the clotting activity of plasma factors, correlated positively to HCT. This observation is in line with a previous study on canine blood (Smith et al. 2009) but is in contrast to a study on equine coagulation (Paltrinieri et al. 2008). This correlation was also supported by the shorter aPTT in the calves.

Analysis of the CV demonstrated good instrument precision, as previously described in horses (Paltrinieri et al. 2008). The lowest CVs were observed for the na-TEM® profile, which represents the coagulation process without the use of activators and is characterized by high individual variability.

Overall, TEM was found to be an accurate device that can be applied in the field to study hemostasis in cattle.

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Chapter 16
Preliminary Evaluation of M-Mode, B-Mode, and X-Strain® Echocardiographic Indices Before and During Therapy in Dogs Affected by Dilated Cardiomyopathy

A. Fruganti, M. Cerquetella, I. Copponi, A. Spaterna, and B. Tesei

Abstract Clinical examination and m-mode, b-mode, and two-dimensional speckle tracking evaluations with X-Strain® software were carried out in two dogs with dilated cardiomyopathy before and during therapy. During follow-up, the two dogs presented not only in better clinical condition, but also with improvements in m-mode and b-mode indices, as well as increased values of strain and strain rate. Consequently, X-Strain® evaluation should be included in routine echocardiography to better understand the pathogenesis and evolution of disease characterized by reduced myocardial contractility, optimize the therapeutic protocol during follow-up, and contribute to finding the range of normal values for strain and strain rate in the dog.

Keywords Dilated cardiomyopathy • Dog • Echocardiography • Strain • Strain rate

16.1 Introduction

Echocardiography is the most valid technique for the evaluation of myocardial function, both in humans and in veterinary medicine. In detail, ultrasonography allows an estimation of ventricular contractility and the dimensions of the cardiac chambers through an evaluation of different specific indices. Nevertheless, it should be considered that "traditional" ultrasonographic techniques (i.e., m-mode and b-mode) do not allow a correct estimation of the complex mechanism of cardiac contraction. During systolic and diastolic activities, because of the particular disposition of the bundles, the myocardial motion is indeed performed in three different directions: radial, longitudinal, and circumferential (Buckberg 2002). The "traditional" ultrasound techniques allow an evaluation of the morphological radial

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and longitudinal changes of the left ventricle during the cardiac cycle; however, the same cannot be said for the study of the circumferential cardiac movement of torsion. In recent years, mainly in human medicine (Thomas and Popovic 2006) but also in veterinary medicine (Estrada and Chetboul 2006; Schwarzwald et al. 2009), two new parameters, strain and strain rate, which are able to quantify a percentage of myocardial displacement, have been taken into consideration. Such indices are obtained through tissue Doppler imaging (TDI) or 2D speckle tracking (2DST). In comparison with TDI, 2DST is independent from the Doppler angle of incidence and appears to be more promising for the evaluation of both regional and global cardiac motion, not only radial and longitudinal but also circumferential (Schwarzwald et al. 2009; D'Andrea et al. 2009). Therefore, the aim of the present study was to verify whether the application of 2DST, through the software X-Strain® of Esaote, allows the making of some considerations inherent to variations of strain and strain rate in dogs affected by left ventricle reduced contractility, prior to and after the beginning of treatment. Furthermore, eventual variations of both m-mode and b-mode parameters will be considered. The eventual positive result of this variation could justify the necessity to comprehend the application of X-Strain® in veterinary medicine during echocardiographic examination. In fact, it could be useful not only to study in more detail the pathogenesis and evolution of diseases characterized by alterations of myocardial contractility but also to optimize therapeutic protocol during patient follow-up and to individualize some normal values of the X-Strain® indices that are currently unavailable for dogs.

16.2 Materials and Methods

A crossbred male dog, 45 kg, 9 years old (case 1), and a Harlequin male Great Dane, 68 kg, 5 years old (case 2), both with an anamnesis of cough and exercise intolerance, underwent physical and echocardiographic examination before (T_0) and 16 weeks and 15 days (respectively) after (T_1) the diagnosis of dilated cardiomyopathy and the consequent beginning of therapy with inotropic drugs (i.e., Pimobendan 0.25 mg/kg per os every 12 h and Digoxin 0.005 mg/kg per os every 12 h, respectively), Furosemide (2 mg/kg per os every 12 h), and Benazepril (0.25 mg/kg per os every 24 h). m-Mode, b-mode, and 2DST evaluations were performed after trichotomy, degreasing, and ultrasound gel application on the right and left parasternal views, with the patient initially in the right and then in the left recumbency. The ultrasound examinations were performed with the echograph Esaote "MyLab30-Vet," with the software X-Strain® and a multifrequency-phased array probe (1–4 MHz). Therefore, for each patient at T_0 and T_1, the following m-mode and b-mode parameters were estimated: interventricular septum at end-diastole (IVSED) and at end-systole (IVSES), end-diastolic (LVEDD) and end-systolic (LVESD) left ventricle diameter, left ventricle posterior wall at end-diastole (LVWED) and at end-systole (LVWES), interventricular septal thickening

fraction (IVSTF), left ventricle posterior wall-thickening fraction (LVWTF), shortening fraction (SF), ejection fraction (EF), left ventricle sphericity index (SI), and left atrium to aorta ratio (LA/Ao). The 2DST evaluations were performed at T_0 and T_1 in each patient with the software X-Strain® after the acquisition of video clips, including three cardiac cycles per patient, from the right parasternal short-axis view of the left ventricle at the level of the papillary muscles. On the three acquired cardiac cycles, X-Strain® allows analysis of the motion of the segments between points that are fixed on the epicardial and endocardial borders of the left ventricle using special bright reference markers on the two-dimensional image. A special rule of the software was applied, in addition, to set the points to standardize the dimensions of the different myocardial angular sectors to be analyzed. Therefore, the motion of the left ventricle was studied and subdivided in six specific sectors as suggested by Lang et al. (2005). For each cardiac sector, X-Strain® allows estimations of two parameters, strain (S) and strain rate (SR). S quantifies the percentage of myocardial deformation caused by the lengthening or shortening of the muscular fibers. It is calculated through the application of the following equation: $S = (L - L_0)/L_0$, where L is the final length of the myocardial segment and L_0 is the initial length. SR, instead, quantifies the velocity of myocardial deformation; it is measured in 1/s (s = second) and is calculated through the application of the following equation: $S/\Delta t$, where S is the strain (percentage of deformation) of the considered myocardial segment and Δt is the duration of the deformation of the same segment. Thus, X-Strain® allowed estimations of the S and SR values of the short-axis of the left ventricle automatically, relative to the radial and circumferential motions in a single cardiac cycle, as the average of the values obtained in each of the six myocardial sectors. Therefore, the mean values over three cardiac cycles of the following indices were evaluated: radial S (rad S), radial SR (rad SR), circumferential S of epicardium (epi circ S) and of endocardium (endo circ S), and circumferential SR of epicardium (epi circ SR) and of endocardium (endo circ SR). The rotational displacement of epicardium (epi rot Displ) and of endocardium (endo rot Displ) and the radial displacement of epicardium (epi rad Displ) and of endocardium (endo rad Displ) were also estimated with X-Strain®.

16.3 Results

The physical examination with particular attention to the cardiovascular system performed in each patient at T_0 allowed detection of weakness with tendency to decubitus, dyspnea at rest, tachisfigmia (160 bpm in case 1 and 150 bpm in case 2), weak pulse, crackles specifically in the middle and cranial pulmonic fields, and a grade 3/6 systolic cardiac murmur loudest over the mitral valve. The clinical examination performed in the same patients at T_1 allowed evaluation of a lower degree of weakness, the disappearance of dyspnea, an increase in the width and reduction in the frequency of the pulse (95 bpm in case 1 and 125 bpm in case 2),

Table 16.1 Results of m-mode and b-mode evaluations performed at T_0 and T_1 in each patient

M- and B-MODE		IVSED (mm)	LVEDD (mm)	LVWED (mm)	IVSES (mm)	LVESD (mm)	LVWES (mm)	IVSTF (%)	LVWTF (%)	SF (%)	EF (%)	SI (%)	LA/Ao
Case 1	T_0	11.6	70.6	10.5	13	65.9	11.6	12	10	7	14	1.04	2
	T_1	13.8	68.8	12	15.6	61.6	13.8	13	15	11	22	1.28	1.4
Case 2	T_0	13.1	65.3	11.6	14.1	59.5	12	8	3	9	19	1.15	1.9
	T_1	10.5	62.3	10.5	12.1	56.3	11.8	15	12	10	21	1.21	1.6

Table 16.2 Results of X-Strain® evaluations performed at T_0 and T_1 in each patient

X-Strain®		Rad S (%)	Rad SR (1/s)	Endo circ S (%)	Endo circ SR (1/s)	Epi circ S (%)	Epi circ SR (1/s)	Endo rot Displ (°)	Endo rad Displ (mm)	Epi rot Displ (°)	Epi rad Displ (mm)
Case 1	T_0	5.61	1.13	−3.34	−0.5	−2.48	−0.37	1.29	1.4	0.61	1.29
	T_1	12.67	1.37	−6.83	−0.65	−3.73	−0.43	2.36	2.19	0.85	1.95
Case 2	T_0	11.9	1.24	−5.42	−0.42	−2.76	−0.32	0.13	2.16	0.48	1.26
	T_1	19	1.42	−6.89	−0.65	−2.8	−0.32	0.87	2.66	1.06	1.4

and the absence of pulmonic crackles; however, the same cardiac murmur that was auscultated at T_0 persisted. The results of the m-mode, b-mode (Table 16.1), and X-Strain® ultrasound evaluations (Table 16.2), performed at T_0 and T_1 in each patient, are reported in the indicated tables.

16.4 Discussion

The clinical and instrumental examinations performed in these two patients allowed us to properly diagnose and choose the right treatment. Therapeutic success was proven not only through ameliorating the clinical conditions of the subjects at T_1 but also by the improvement in the echocardiographic indices at the same time. Together, the m-mode, b-mode, and X-Strain® indices proved a reduction in the internal dimensions of the left cardiac chambers and an increase in myocardial contractility. In detail, the increase at T_1 of the X-Strain® parameters, such as the epi circ S, endo circ S, epi circ SR, and endo circ SR, also revealed improvement in the circumferential movement of the left ventricle. In fact, torsion could not be studied through the application of traditional mono- and two-dimensional techniques. Although preliminary, the results of the present study suggest the importance of including X-Strain® in routine echocardiographic evaluations of patients affected by alterations in myocardial movement. Despite an increase in examination time, the inclusion of X-Strain® in medical practice could be useful both to better understand the pathogenesis of diseases characterized by alterations in myocardial contractility and to increase the accuracy of patient monitoring during follow-up to optimize the therapeutic protocol as well as to identify the reference limits of X-Strain® indices in the dog.

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Chapter 17
Pharmacokinetics of the Tramadol Injective Formulations in Alpacas (*Vicugna pacos*)

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Abstract The objective of the present study was to test the intravenous (IV) and intramuscular (IM) administration of tramadol to alpacas (*Vicugna pacos*). The study was a two-group, single-dose, two-treatment, two-period, randomized, open, balanced, cross-over design. After both administrations of tramadol to alpacas (2.5 mg/kg b.w.), plasma concentrations of tramadol and its main metabolites (M1, M2, and M5) were determined using an HPLC method. Moderate side effects were shown after IV administration. The IM bioavailability of the drug (81.5%) was within the generally accepted values for a positive bioequivalence decision (80–125%). The plasma concentrations of M1 and M5 were low, and the amounts of M2 produced were similar after both routes of administration. In conclusion, tramadol was rapidly and almost completely absorbed after IM administration, and its systemic availability was equivalent to IV injection. Hence, IM injection of tramadol is a useful and good alternative to IV injection in alpacas.

Keywords Tramadol • Alpaca • Intravenous • Intramuscular • Pharmacokinetics

17.1 Introduction

Tramadol is a centrally acting analgesic drug that has been used clinically for the last two decades to treat pain in humans. Recently, tramadol was reported to be metabolized into inactive metabolites faster in dogs (KuKanich and Papich 2004;

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Giorgi et al. 2009a, b, c), goats (de Sousa et al. 2008), and horses (Giorgi et al. 2007) than in humans (Grond and Sablotzki 2004). The clinical effectiveness of this drug has been questioned in the animal species in which the drug is mainly metabolized into inactive substances. The animal species seem to be related to both the bioavailability of tramadol and the metabolic patterns leading to differing amounts of M1 (KuKanich and Papich 2004; de Sousa et al. 2008; Giorgi et al. 2007, 2009a, b, c). In dromedary camels (Elghazali et al. 2008), a single study reported plasma concentrations of tramadol, but no data were reported for its active metabolite M1 in plasma. Therefore, the aim of the present study was to test the intravenous (IV) and intramuscular (IM) administration of tramadol in alpacas (domesticated species of South American camelid) to assess its pharmacokinetic profile.

17.2 Materials and Methods

Eight healthy male alpacas (*Vicugna pacos*), aged 5–9 years and weighing 41–58 kg, were used. The animals were previously determined to be clinically healthy based on a physical examination and full hematological analyses. The study protocol, conforming to EU regulations (86/609/EEC), was approved by the ethics committee of the University of Pisa, Italy (authorization no. 9403).

Test preparations were made according to an open balanced cross-over design: animals were assigned to two treatment groups, using a single dose and two periods. Each animal received a single dose of 2.5 mg/kg tramadol (Altadol; Formevet, Milan, Italy) via IV or IM. A catheter was placed into the left jugular vein to facilitate slow IV drug administration (2 min). The IM injection was placed in the upper outer quadrant of the buttocks. After the wash-out period of 2 weeks, the groups were inverted. By another catheter placed into the right jugular vein, blood samples (5 mL) were collected at 0, 5, 15, 30, and 45 min, and 1, 1.5, 2, 4, 6, 8, 10, and 24 h after administration of tramadol into collection tubes containing lithium heparin. The blood samples were centrifuged at 3,000 rpm within 30 min after collection, and the harvested plasma was stored at −20°C until use within 30 days of collection (de Sousa et al. 2008).

The concentrations of tramadol, M1, M2, and M5, in plasma were evaluated using HPLC according to the method described by Giorgi et al. (2007). The HPLC system was an LC Workstation Prostar (Varian Corporation, Walnut Creek, CA) consisting of an LC Prostar 230 pump, combined with an RF-10A spectrofluorometric detector. Excitation and emission wavelengths were 275 and 300 nm, respectively. Chromatographic separation was performed twice on a Luna C18 ODS2 analytical column (150 mm × 2.1 mm i.d., 3-μm, Phenomenex, Bologna, Italy) maintained at 23 ± 0.5°C with a flow rate of 0.7 mL/min. The mobile phase consisted of metanolo/formic acid (99:1 v/v) and buffer (5 mM ammonium acetate, adjusted to pH 4.5 with acetic acid; 40:60 v/v). The pharmacokinetic calculations were performed using the WinNonLin v. 5.2.1 program (Pharsight Corp., Cary, NC).

17.3 Results

A few minutes after tramadol IV administration, one of the subjects had an epileptic crisis that lasted 3 min. When the same dose was administered slowly (within 2 min), only mild sedation and tremors lasting less than 30 min were present in all animals. No adverse effects were noted after IM administration of the same dose of drug.

The plasma concentrations/time curves of tramadol after IV and IM administration are reported in Fig. 17.1. A noncompartmental model fitted the plasma concentrations of tramadol. The elimination half-life, the volume of distribution, and the systemic clearance of tramadol were 1.20 ± 0.61 and 0.78 ± 0.25 h, 3.42 ± 2.36 and 1.54 ± 0.54 L/kg, and 2.15 ± 0.35 and 1.29 ± 0.39 L/h/kg, following IV and IM administration, respectively. These values were significantly different ($P < 0.05$) between groups. The mean systemic bioavailability of tramadol administered IM was $81.5 \pm 14.3\%$ with a range of values between 75 and 96%. After IM administration, the time taken to reach the minimal effective concentration (MEC) t_e, and the period of time during which this plasma concentration was exceeded, Δt_e, were 1.0 ± 0.2 min and 2.5 ± 0.35 h, respectively. Following IV administration, Δt_e was 3.15 ± 0.30 h. The M2 metabolite had similar plasma concentration/time curves after both administrations, and it was detectable from 5 min to 4 and 6 h after IV and IM treatment, respectively. By contrast, M1 and M5 were detected in one and two alpacas, respectively. These metabolites were detected at concentrations close to the limit of quantification (LOQ) for the method (0.005 μg/mL). Therefore, their pharmacokinetic parameters

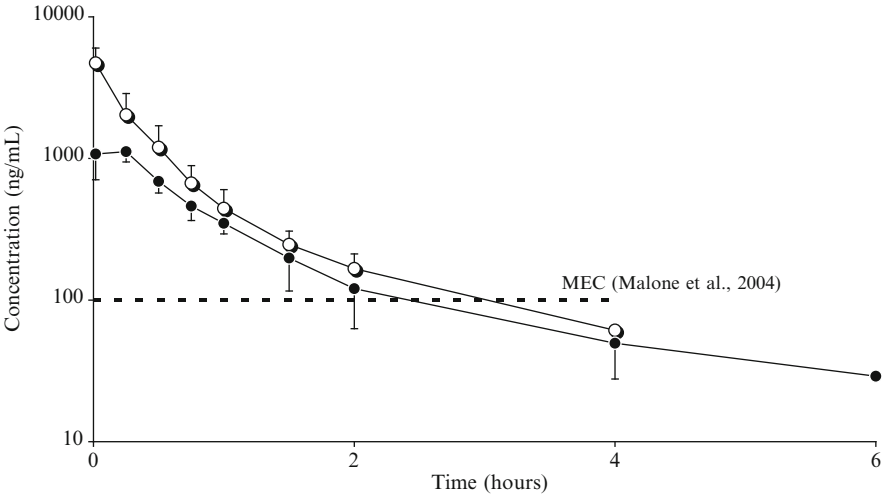

Fig. 17.1 Plasma concentrations/time curves of tramadol following IV (*open circle*) and IM (*closed circle*) administration

were not calculated. The chromatographic curves from both treatments showed some time-dependent unknown peaks, likely metabolites not currently identified.

17.4 Discussion

The administration of tramadol has been widely studied in the recent past in both equine (Giorgi et al. 2007) and ruminant species (de Sousa et al. 2008; Elghazali et al. 2008). M1 concentrations are low in plasma from these species with an assumed lack of effectiveness for pain therapies. This study reports for the first time the plasma concentration of the main metabolites of tramadol (M1, M2, and M5) in the camelid species alpaca (*V. pacos*). To estimate the onset time and the duration of action following IM and IV injection, the clinically relevant therapeutic parameters t_e and Δt_e were calculated for an assumed MEC (Malonne et al. 2004). A target plasma concentration of tramadol of 100 ng/mL was clinically effective in the treatment of mild to moderate pain in humans (Malonne et al. 2004). The Δt_e values for IM and IV administration were similar, with small differences likely due to different initial time courses of absorption.

These data assume that the MEC as calculated for humans is relevant for alpacas and should be integrated with further pharmacodynamic studies. To determine the analgesic effect of tramadol in humans, some authors have used the plasma concentration of M1, the molecule indicated as responsible for most of the therapeutic effects (Garrido et al. 2003). In the present study, the metabolite M1 was detected at a concentration at or lower than the MEC (0.040 ± 0.030 μg/mL) reported for humans (Grond and Sablotzki 2004), and calculation of Δt_e for M1 was not possible. The low concentrations of the active metabolite are in accordance with earlier studies in goats (de Sousa et al. 2008), equines (Giorgi et al. 2007), and dogs (KuKanich and Papich 2004; Giorgi et al. 2009a, b, c), and in contrast to those reported for cats (Pypendop and Ilkiw 2008) and humans (Grond and Sablotzki 2004). The present study reports a higher plasmatic concentration of M2 than of M5 or M1. For the first time, a low plasma concentration of M5 has been reported. This could be due to the remarkable glucuronidation process in camelids (Al Katheeri et al. 2005) that leads to a high elimination rate for M1 and M5 (as glucuronides), without influencing M2.

Although the IV and IM routes of administration are almost bioequivalent ($F = 81.5\%$) (Toutain and Bousquet-Melou 2004), the lower initial plasma concentrations of tramadol following IM administration may be therapeutically beneficial. IM administration has been suggested to have a lower incidence of side effects with a slightly longer onset of action (Lintz et al. 1999). Therefore, according to the data generated in this study, IM injection of tramadol in alpacas is a useful and better alternative to IV injection, despite the lack in the production of M1 that could limit the analgesic effect.

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Chapter 18
Contrast-Enhanced Ultrasonography for Characterization of Liver Lesions in Dogs and Cats

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Abstract Liver nodules are common in dogs. Contrast-enhanced ultrasound (CEUS) is commonly used to characterize focal hepatic lesions in humans. Three vascular phases have been described in the liver: arterial phase, portal phase, and late phase. Contrast-ultrasonographic features of canine liver lesions have also been described. The hypoechogenicity at peak liver enhancement and during the portal phase has been significantly associated with malignancy with high sensitivity and specificity. Few reports on contrast ultrasonographic features of hepatic lesions in cats are available. The aim of this study was to describe contrast ultrasonographic features of 16 liver lesions in 13 dogs and two cats. All patients underwent fundamental abdominal ultrasound, anesthesia, contrast-enhanced ultrasound with a second-generation contrast medium (sulfur hexafluoride-SonoVue®), fine-needle aspiration/tissue core or surgical biopsy of the lesions, followed by cytological/histological examination. Ten lesions were benign (four nodular hyperplasias, three simple cysts, two nodules of focal hydropic degeneration, and one eosinophilic infiltration) and six were malignant (four hepatocellular carcinomas, one biliary carcinoma, and one lymphoma). All benign lesions, with the exception of the simple cysts, were iso- or hyperechoic to the surrounding tissue in the portal phase, while all malignant lesions were hypoechoic. The hypoechogenicity during the portal phase was significantly associated with malignancy with high sensitivity and specificity (100% and 70%, respectively). According to the literature, CEUS of the liver resulted in a safe diagnostic procedure that was of value in differentiating benign from malignant hepatic lesions. CEUS, however, is not able to define tumor cell type, and either biopsy or fine-needle aspiration is recommended to achieve a final diagnosis.

Keywords Contrast • Ultrasound • Liver • Lesion • Dog • Cat

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18.1 Introduction

Nodular parenchymal hepatic lesions are frequent in small animals. Focal nodular hyperplasia is present in 70% of dogs older than 6 years (Bergman 1985). Other less common lesions are represented by hematoma, cyst, abscess, focal necrotic area, primary neoplasm (hepatocellular carcinoma, biliary carcinoma, carcinoids, sarcoma), or secondary neoplasm (hemangiosarcoma, insulinoma, pancreatic carcinoma, fibrosarcoma) (Haers and Saunders 2009). Ultrasonography (US) with second-generation contrast medium, a quite recent diagnostic method, frequently used in human and veterinary medicine, allows the visualization of lesion perfusion in real time. The ultrasonographic features of normal liver vascularity have been described (Ziegler et al. 2003), and three vascular phases have been identified: arterial phase, portal phase, and late phase (Ziegler et al. 2003; O'Brien et al. 2004). The arterial phase is identified 7–10 s after the intravenous injection of the contrast medium and persists for approximately 15 s. Afterward, portal phase is identified after 35–40 s and continues for approximately 120 s. The late phase is determined by the presence of the contrast medium within the hepatic sinusoid and can persist for 4–20 min, depending on the contrast medium employed. The pathological modifications of hepatic vascularity during the three mentioned vascular phases are important for the characterization of hepatic lesions. Malignant lesions can be differentiated from benign lesions because the former have an early arterial phase and poor to absent portal and late phases compared to normal liver parenchyma (Ziegler et al. 2003; O'Brien et al. 2004; Quaia 2007). The hypoechogenicity of nodular hepatic lesions at peak liver enhancement and during the portal/late phases has been significantly associated with malignancy, with high positive and negative predictive values in both human and veterinary medicine (O'Brien et al. 2004; Quaia 2007). The aim of this study was to report our experience with the characterization of benign and malignant nodular hepatic lesions in the dog and cat with contrast-enhanced US and to compare our data with those published in the literature.

18.2 Materials and Methods

Thirteen dogs of different breeds and sexes, from 5 to 13 years old, and two cats (mixed breeds, a male and a female, 7 and 10 years old respectively), with single or multiple nodular hepatic lesions were considered. Each animal underwent abdominal US, anesthesia, contrast US of the nodular hepatic lesions, ultrasound-guided fine needle aspirate/biopsy or surgical removal of the hepatic lesions, and cytological/histological examination of the collected specimen. Contrast-enhanced US (CEUS) has been performed using two different ultrasonographic units and the CnTI harmonic technique (Esaote® Megas GPX and Esaote® MyLab 30 GOLD). Two different probes were employed: a convex probe with a receiving frequency of

3.2 MHz and a linear probe with a receiving frequency of 5 MHz. A focal zone was always set at the level of the deepest portion of the lesion to be examined. The mechanical index was always less than 0.1 (acoustic pressure <45 kPa). A bolus intravenous injection of the contrast medium composed of sulfur hexafluoride encapsulated by a shell of phospholipids (SonoVue®-Bracco) at a dosage of 0.03 ml/kg b.w. into the cephalic vein, followed by a saline flush at a dosage of 5 ml for the dogs and 2.5 ml for the cats, was performed. Regional blood perfusion was visualized in the region of interest for 2 min after intravenous injection. Tissue echogenicity of the considered liver lesions was evaluated during the arterial phase, peak liver enhancement, and portal phase and was then compared with normal surrounding liver tissue. Each diagnostic examination was recorded digitally. The collected data from the region of interest were then elaborated using commercial software (*Qontrast®-Bracco*), and time–intensity curves were constructed using the gamma variate model. The peak intensity P (percentage of the signal intensity, SI), time to peak in seconds (TTP), regional blood flow RBF (ratio between the regional blood volume and the medium transit time), and the medium transit time (MTT) in seconds were calculated. Contrast-enhancement patterns during the arterial and portal phases of the benign and malignant lesions were studied with Fischer's exact test. Values for $P < 0.05$ were considered significant.

18.3 Results

Sixteen hepatic focal lesions were evaluated: 12 were single and four were multiple. Ten lesions were described as benign: nodular hyperplasia (four cases), hydropic degeneration (two cases), simple cystic lesions (three cases), and eosinophilic infiltrate (one case). The other six lesions were malignant: hepatocellular carcinoma (four cases), biliary carcinoma (one case), and lymphoma (one case). In one patient, two different types of lesions were present. One benign lesion (cyst) and one malignant lesion (hepatocellular carcinoma) belonged to the two feline patients. Two cases of nodular hyperplasia were hypoechoic and two were hyperechoic by fundamental US. Hydropic degeneration nodules were always hypoechoic. Hepatic cysts appeared as anechoic cavitary lesions with a thin capsule, characterized by distal enhancement. Eosinophilic infiltrate was characterized by multiple, small, hypoechoic, poorly defined nodules. Among the malignant lesions, two hepatocellular carcinomas were hypoechoic, while the other two showed a mixed echotexture. Biliary carcinoma appeared as multiple nodular target lesions, and lymphoma appeared as a hypoechoic focal lesion.

During CEUS examination, two cases of nodular hyperplasia were hyperechoic during the arterial phase, while the other two were hypoechoic. During the portal phase, three of these were isoechoic, while one was hyperechoic (data media: P: 36.7%, TTP: 49.6 s, RBF: 45.9, MTT: 80.7 s; surrounding hepatic tissue: 34.7%, TTP: 53.5 s, RBF 43, MTT: 82 s). One case of hydropic degeneration appeared hyperechoic during the arterial phase, while the other was hypoechoic.

At peak liver enhancement, both hydropic degenerations were slightly hypoechoic to the surrounding hepatic tissue. During the portal phase, they both appeared isoechoic to the surrounding tissue (data media: P: 52.1%, TTP: 54 s, RBF: 66.9, MTT: 96 s; surrounding tissue: P: 53.4%, TTP: 53.9 s, RBF: 65.8, MTT: 94.9 s). Cystic hepatic lesions always appeared anechoic during the arterial and portal phases. The collected data relative to the eosinophilic infiltrate were similar to the data of the surrounding tissue (data collected for the nodular lesion: P: 43.6%, TTP: 31.8 s, RBF: 59, MTT: 66.2 s; surrounding tissue: P: 40.4%, TTP: 38.6 s, RBF: 52.6, MTT: 65.2 s). Among the hepatocellular carcinomas, three were hyperechoic during the arterial blood phase (two dishomogeneous and one homogeneous) and one was hypoechoic. At peak liver enhancement, three were hypoechoic and one was isoechoic to the surrounding hepatic tissue. All appeared hypoechoic during the portal blood phase (data media: P: 28.6%, TTP: 73.4 s, RBF: 36.7, MTT: 54.1 s; surrounding hepatic tissue: P: 78%, TTP: 49.3 s, RBF: 42.6, MTT: 92.6 s). Biliary carcinoma was hyperechoic during the arterial blood phase and hypoechoic at peak liver enhancement and during the portal blood phase (data media: P: 44.8%, TTP: 30.4 s, RBF: 59.8, MTT: 58.2 s; surrounding hepatic tissue: P: 50.4%, TTP: 47 s, RBF: 66, MTT: 85.4 s). The lymphoma was isoechoic during the arterial blood phase and hypoechoic at peak liver enhancement and during the portal blood phase (data media: P: 29.8%, TTP: 31.7 s, RBF: 34.8, MTT: 44.6 s; surrounding hepatic tissue: P: 36.5%, TTP: 73.6 s, RBF: 44.4, MTT: 105.7 s). Significant differences between the ultrasonographic features of the benign/malignant lesions during the arterial blood phase and at peak liver intensity were not observed. A statistically significant difference in echogenicity during the portal blood phase of the benign and malign nodular hepatic lesions was described. All malignant lesions were hypoechoic during the portal and late phase as, with the exception of the simple cysts, all the benign lesions were hyper/isoechoic to the surrounding hepatic tissue. The hypoechogenicity during the portal phase, as an important feature for the discrimination between benign and malignant hepatic nodular lesions, showed a sensitivity and specificity, respectively, of 100% (CI 95%: 100–100) and of 70% (CI 95%: 41.6–98.4), for a positive predictive value of 66.7% (CI 95%: 35.9–97.5) and a negative predictive value of 100% (CI 95%: 100–100).

18.4 Discussion

CEUS is routinely employed in human medicine to detect and characterize focal hepatic lesions (Quaia 2007). The most significant feature of malignancy is hypoechogenicity during the portal phase, possibly due to new arterial blood supply associated with a concomitant reduction of portal blood vessels (Ziegler et al. 2003; Quaia 2007). In veterinary medicine, CEUS has been widely employed for the characterization of hepatic lesions in the dog (O'Brien et al. 2004; Ivančić et al. 2009; O'Brien 2007; Volta et al. 2009) as few studies have been done in the cat

(Webster and Holloway 2008). In addition, CEUS has been very helpful for the detection of metastatic liver nodules of hemangiosarcoma, which is undetectable with B-mode US (O'Brien 2007). In the dog, the hypoechogenicity of nodular hepatic lesions at the peak liver enhancement was significantly associated with malignancy, showing high sensitivity (100%), specificity (94.1%), positive predictive value (93.8%), and negative predictive value (100%) (O'Brien et al. 2004). The data collected in this study regarding hepatic malignant focal lesions are similar to those reported in the literature, with the exception of hepatocellular carcinoma in the cat, which was isoechoic at peak liver enhancement (arterial blood phase) when compared to the surrounding normal liver parenchyma, and became hypoechoic during the portal blood phase. The echogenicity of the lesion at peak liver enhancement was not statistically significant to discriminate a benign or malignant origin. The hypoechogenicity during the portal blood phase was the only ultrasonographic feature that was related to malignancy. Its sensitivity and specificity were quite high, according to the literature in human medicine (Quaia 2007; Nyman et al. 2004). CEUS is always well tolerated without any side effects. Lesions' perfusion was accurately visualized in all cases. In two cases of hepatocellular carcinoma, it was possible to identify other hypovascular lesions during the portal phase, which were not visible during the B-mode ultrasonographic examination.

In conclusion, CEUS of nodular hepatic lesions can be considered a diagnostic method that is easy to perform, minimally invasive, and well tolerated. The evaluation of the hemodynamics of the contrast medium during the vascular perfusion of the hepatic lesions improves the diagnostic accuracy of US in the study of malignant lesions (Haers and Saunders 2009; Quaia 2007; Nyman et al. 2004). In conclusion, lesions that are hypoechoic during peak liver enhancement and, in particular, during the portal phase are significantly associated with malignancy (primary hepatic neoplasia/metastasis) (O'Brien et al. 2004; Quaia 2007). However, in veterinary medicine, CEUS is unable to define clearly the cellular type of neoplasia, due to the deficiency of reported studies on this topic in the literature. Therefore, cyto/histological studies are still needed to make a final diagnosis in cases of malignancy.

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Chapter 19
Canine Chronic Kidney Disease: Retrospective Study of a 10-Year Period of Clinical Activity

G. Guidi, C. Rossini, C. Cinelli, V. Meucci, and I. Lippi

Abstract Chronic kidney disease (CKD) is a progressive, pathological disease with a higher prevalence in geriatric patients. The aim of the present study was to estimate the prevalence of dogs affected by CKD at the Department of Veterinary Clinics over a 10-year period of clinical activity, according to gender, age, breed, and IRIS stage. Males showed a prevalence of 60.17%, while females showed a prevalence of 39.82%; 28.40% of dogs were younger than 6 years, 42.12% were between 6 and 10 years, and 29.46% were older than 10 years. Mixed breed patients showed a prevalence of 31.38%, followed by Boxer (9.36%), German shepherd (7.76%), and setter (5.42%). The prevalence of CKD was 24.25% in IRIS 2, 52.97% in IRIS 3, and 22.76 in IRIS 4. The results showed that CKD represents a more frequent reason for consultation in canine patients than in the past. It is not clear whether the high prevalence of CKD is due to an actual increase in the disease or an increased frequency of early diagnosis.

Keywords Chronic kidney disease • Dog • IRIS • Prevalence

19.1 Introduction

Chronic kidney disease (CKD) is a complex clinical syndrome that represents the end point of a progressive pathological process of one or more sections of the nephron (glomerule, tubule, interstice, and vessel) (Brown 2007). The small number of epidemiologic studies concerning the prevalence of CKD in dogs reflects the situation in the United States. CKD was prevalent in 0.5–1.5% of the dogs

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presented for clinical consultation and reached 10% in geriatric patients (Brown et al. 1998; Grauer 2009). At present, no epidemiological data concerning the prevalence of CKD in dogs according to the International Renal Interest Society (IRIS) staging system, gender, age, or breed are available. The aim of the present study was to evaluate the prevalence of CKD in a canine population presented for clinical consultation to the Veterinary Teaching Hospital between 2000 and 2009 based on IRIS stage, gender, age, and breed.

19.2 Materials and Methods

All dogs submitted for clinical consultations at the Veterinary Teaching Hospital between January 2000 and December 2009 (3,764 patients: 1,749 females and 2,015 males) were enrolled in the present study. CKD dogs (940 subjects) were identified based on history, clinical evaluation, and plasma creatinine levels (creatinine >1.4 mg/dL). Patients were classified according to plasma creatinine levels into different IRIS stages (IRIS 2: 1.4–2.0 mg/dL; IRIS 3: 2.1–5.0 mg/dL; IRIS 4: >5 mg/dL), and divided by gender, breed, and age (group 1: ≤5 years old; group 2: 6–10 years old; and group 3: >10 years old). The prevalence of CKD dogs within the total canine population presented for clinical evaluation, the prevalence of CKD in different IRIS stages, and the relationship of gender, age, and breed with CKD prevalence was evaluated.

19.3 Results

Among the 3,764 patients, 940 were determined to be affected by CKD (Fig. 19.1) with a prevalence of 24.97%. The prevalence of CKD in females (375 dogs) was 39.82%, while the prevalence in males (565 dogs) was 60.17%. The prevalence of CKD based on age was 28.40% in group 1 (267 dogs), 42.12% in group 2 (396 dogs), and 29.46% in group 3 (277 dogs; Fig. 19.2). Among different breeds, mixed-breed dogs had the highest prevalence of CKD (31.38%), followed by boxers (9.36%), German shepherds (7.76%), and setters (5.42%; Fig. 19.3). The remaining 30.75% of patients were composed of other breeds with a prevalence

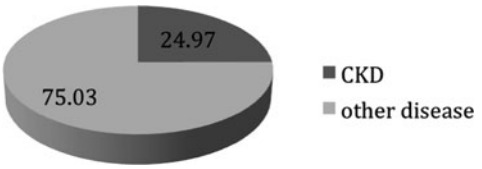

Fig. 19.1 Prevalence of CKD in the canine population presented for clinical consultation to the Veterinary Teaching Hospital between 2000 and 2009. The prevalence of CKD was 24.97%, while 75.03% of patients were affected by other diseases

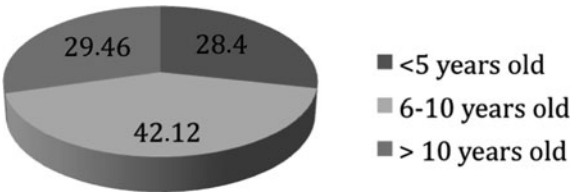

Fig. 19.2 Prevalence of CKD patients according to age (group 1, group 2, and group 3). The prevalence of CKD was 28.4% in group 1 (<5 years old), 42.12% in group 2 (6–10 years old), and 29.46% in group 3 (>10 years old)

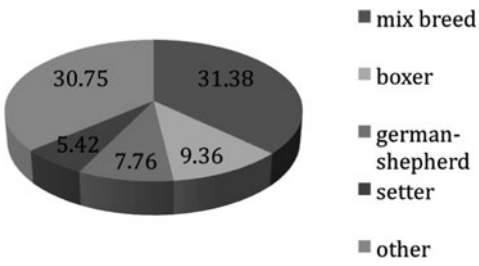

Fig. 19.3 Prevalence of CKD in different canine breeds. Mixed-breed dogs showed the highest CKD prevalence (31.38%), followed by boxers (9.36%), German shepherds (7.76%), and setters (5.42%). The remaining 30.75% of patients was composed of other breeds with a prevalence less than 5%

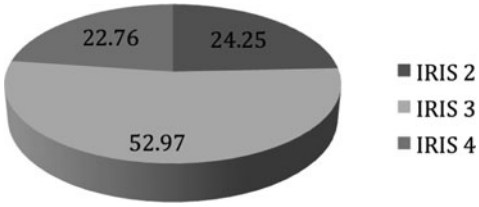

Fig. 19.4 Prevalence of CKD based on IRIS stages. IRIS stage 2 showed a prevalence of 24.25%, while the prevalence for IRIS stages 3 and 4 was 52.97% and 22.76%, respectively

of less than 5%. Based on plasma creatinine levels, 228 dogs were in IRIS stage 2 (24.25%), 498 in IRIS stage 3 (52.97%), and 214 in IRIS stage 4 (22.76%; Fig. 19.4).

19.4 Discussion

The present study showed that the prevalence of CKD in the canine population presented for clinical consultation to the Veterinary Teaching Hospital between 2000 and 2009 was 24.97%, despite the significantly lower prevalence (0.5–1.5%)

reported previously. The high prevalence of CKD found in this study may be due both to the application of more accurate diagnostic procedures and to a greater sensitivity of pet owners to CKD. The elevated prevalence of CKD in our population of dogs may also have been influenced by the fact that the Veterinary Teaching Hospital is a referring center for renal diseases.

The significantly higher prevalence (52.97%) of CKD in IRIS stage 3 patients as compared to IRIS stage 2 and 4 patients may be due to the more evident symptomatology that was easily recognized by the pet owners. The elevated prevalence of IRIS stage 3 dogs may also reflect a chronic trend of the disease and an ability of CKD to develop a balance between the patient and the disease.

Although the present results are similar to previous data that showed a higher prevalence of CKD in advanced age subjects, here, the highest prevalence of disease (42.12%) was found in group 2 (6–10 years old). Difficulties in finding hemodialysis centers and the attention of veterinarians and owners to CKD have increased the use of early diagnosis tests even in nongeriatric patients. Although CKD is more typical in advanced age, the significantly higher prevalence of the disease (28.40%) in group 1 subjects ($\leq$5 years old) should be further investigated to evaluate the prevalence of congenital and acquired types.

Data concerning the prevalence of CKD in different breeds indicated the highest prevalence of CKD to be in mixed-breed (31.38%) dogs, followed by boxers, German shepherds, and setters (each with a prevalence of greater than 5%). Although mixed-breed dogs can include both small and large subjects, the significantly elevated prevalence of CKD in boxers, German shepherds, and setters may reflect a major prevalence of renal disease in medium and large dogs. Although the present data showed that CKD represents a more frequent reason of consultation as compared to that reported previously, it remains unknown if this is caused by an effective increase in prevalence or by more accurate diagnostic procedures.

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Chapter 20
Doppler Echocardiographic Prediction of Pulmonary Hypertension in Canine Leishmaniasis

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Abstract Ten symptomatic dogs, naturally infected by *Leishmania infantum*, were examined by means of Doppler echocardiography to identify the presence of pulmonary arterial hypertension (PAH). The presence of two-dimensional features of PAH and the presence of tricuspid and/or pulmonic valve regurgitation for pulmonary artery pressure quantification were investigated, and standard echo-Doppler variables (i.e., AT, AT/ET, and Tei) were calculated. None of the examined dogs showed two-dimensional features of PAH. In only one dog with mitral endocardiosis, PAH was diagnosed by tricuspid jet velocity. Echo-Doppler variables were not indicative of PAH in all dogs except one, which had a Tei index value predictive of PAH but normal AT and AT/ET. Our preliminary data do not support the presence of PAH in canine leishmaniasis; however, the study population must be increased to yield stronger conclusions.

Keywords Dog • Doppler echocardiography • *Leishmania infantum* • Pulmonary hypertension

Abbreviations

CanL Canine leishmaniasis
PAH Pulmonary arterial hypertension

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20.1 Introduction

Chronic interstitial pneumonitis has been described in both human and canine leishmaniasis (CanL) in the absence of specific respiratory symptoms. In dogs, diffuse interstitial pneumonitis is also associated with arterial obstruction and has been reported in experimental (Keenan et al. 1984) and natural *Leishmania infantum* infections (Duarte et al. 1986; Tafuri et al. 2001). Histopathological findings of chronic and diffuse pneumonitis associated with fibrosis have been observed in dogs naturally infected with *Leishmania chagasi* (*sin. L. infantum*) and were included in a study conducted in Brazil. In this study, the presence of pulmonary lesions was correlated with the disease independent of the presence and severity of clinical signs (Goncalves et al. 2003). In addition, specific respiratory symptoms have not been reported in CanL, and there are no references about the correlation between interstitial pneumonitis and clinical status (Goncalves et al. 2003). Recently, cardiac and pulmonary alterations in dogs naturally infected with *L. chagasi* have been investigated by means of histopathological, cytological, and immunohistochemical analyses (Alves et al. 2010). Inflammatory lesions in pulmonary tissues were mostly focal and not diffuse as previously reported, and, upon cytological examination, the presence of amastigotes in lung imprints was revealed in only 2 of 22 examined dogs (Alves et al. 2010).

Chronic pulmonary diseases may lead to pulmonary arterial hypertension (PAH), a serious progressive pathological condition that affects the subject's quality of life and exercise capacity, which has a poor prognosis if not identified and treated early. PAH clinical signs are vague and aspecific and usually correlate to the primary causative disease. Pulmonary vasoconstriction in response to alveolar hypoxia and tissue acidosis is the most important mechanism of PAH secondary to chronic pneumonitis; the exiting muscular hypertrophy and intimal fibrosis of pulmonary arteries further complicate the hypertensive status (Johnson et al. 1999; Glaus et al. 2003).

The gold standard method for diagnosing PAH is cardiac catheterization. Nevertheless, Doppler echocardiography can be used for noninvasive predictions of systolic and/or diastolic pulmonary artery pressure starting by the measurements of maximal velocity of regurgitant flow across the tricuspid or pulmonic valve, respectively (Johnson et al. 1999). Other Doppler-derived PAH markers have been proposed, including the right ventricular acceleration time (AT), the acceleration time index that is the AT/right ventricular ejection time ratio (AT/ET) (Shober and Baade 2006), the right ventricular Tei index that is an index of myocardial performance (Teshima et al. 2006; Vonk et al. 2007), the pulmonary velocity profile examination (Johnson et al. 1999; McQuillan et al. 2001), and recently tissue Doppler imaging, an ultrasound technique that allows quantitative assessment of segmental myocardial motion (Serres et al. 2007).

The pulmonary lesions observed in human and canine leishmaniasis may result in PAH development, but until now no data was available to support the presence of PAH in *L. infantum*-infected dogs or the correlation between the histopathological

pulmonary findings and clinical status. The aim of this study was to evaluate the risk for developing PAH in symptomatic dogs naturally infected with *L. infantum* living in an endemic area by means of doppler echocardiography. In particular, in the absence of a regurgitant flow across the tricuspid and/or pulmonic valve, indirect-PAH markers were evaluated, including the AT, AT/ET, right ventricular Tei index, and pulmonary velocity profiles.

20.2 Materials and Methods

Dogs of different ages, sexes, and breeds referred at the Veterinary Teaching Hospital of the University of Bari (Italy) with a diagnosis of *L. infantum* infection as determined by microscopic detection of amastigotes on lymph-nodal smears were selected for this study. A clinical examination was performed on each dog, and blood samples were collected during routine laboratory exams. A conventional echocardiographic and Doppler examination was performed on all selected animals. An Esaote Mylab 30 ultrasonic diagnostic apparatus (Medmark-Esaote, Italia) equipped with a 2.5–3.5 MHz cardiologic probe was used. All ultrasonographic examinations were registered and the presence of two-dimensional findings of PAH as well as the presence and velocity of tricuspid and/or pulmonary valve regurgitant jets for systolic and diastolic pulmonary arterial pressure estimation were reported.

In the absence of echocardiographic findings indicative of moderate to severe PAH and in the absence of discernable tricuspid and/or pulmonary valve regurgitant jets, the AT index was determined and the AT/ET ratio was calculated to estimate pulmonary artery pressure. Furthermore, the Tei index was calculated and the pulmonary artery Doppler flow pattern profile was evaluated and classified as previously described (Johnson et al. 1999). The pulmonary flow velocity profile and the AT and ET measurements were recorded from the standard right parasternal short axis view at the basal level. AT and ET values were measured from the onset of pulsed Doppler flow to peak flow velocity and from the onset to the end of the pulsed Doppler flow, respectively. The Tei index was calculated using the formula $(I - \mathrm{ET})/\mathrm{ET}$, where I is the interval between cessation and the onset of tricuspid inflow and was assessed using the left apical four-chamber view.

20.3 Results

Preliminary results were obtained from 10 symptomatic dogs with diagnoses of leishmaniasis without previous treatment histories. Two dogs showed a left systolic murmur at cardiac auscultation, and the echocardiographic exam permitted a diagnosis of degenerative mitral valve disease. Neither two-dimensional echocardiographic findings of PAH (i.e., enlarged right ventricle, flattened interventricular

septum, or dilatation of the main pulmonary artery) nor pulmonary valve regurgitant jets were registered in any dog. A tricuspid regurgitant jet having a peak velocity of 3.3 m/s was revealed in one of two dogs with mitral valve endocardiosis. In this dog, a systolic pressure value of 48.56 mmHg was calculated using the modified Bernoulli equation. In all dogs, the AT index ranged from 73 to 90 ms and the AT/ET ratio ranged from 0.36 to 0.52. In particular, the AT/ET ratio was higher than 0.42 ms in six dogs. The Tei index ranged from 0.15 to 0.48 with only one dog showing a value higher than 0.36. The pulmonary flow profiles were classified as pattern I in three dogs and pattern II in six dogs.

20.4 Discussion

None of the dogs included in this study showed a condition of severe PAH associated to CanL. In one dog, a Cavalier King Charles Spaniel with mitral valve endocardiosis (ISACH 1), a tricuspid regurgitant jet having a peak velocity of 3.3 m/s, was registered and was considered indicative of PAH. A tricuspid regurgitant jet peak velocity equal to or higher than 2.8 m/s is abnormal and, in the absence of pulmonary stenosis, is indicative of PAH (Johnson et al. 1999). The dog was categorized as having mild PAH since the calculated systolic pressure value was 30–55 mmHg (Pyle et al. 2004). It is unclear whether the PAH registered in this dog was the consequence of mitral valve insufficiency, leishmaniasis, or both conditions. PAH can be identified in dogs with mitral valve degenerative diseases in which the left atrium/aorta ratio is within the reference range (Serres et al. 2006).

Other indirect Doppler echocardiographic markers were used to investigate PAH in this study, because alterations in two-dimensional echocardiograms were registered only during severe PAH and, in the absence of measurable tricuspid or pulmonary regurgitant jets, assessment of pulmonary artery pressure may be impossible (Serres et al. 2007). Echo-Doppler variables, such as AT, AT/ET, and TEI, are highly correlated to systolic pulmonary arterial pressures; thus, any alteration in those measurements may raise the suspicion of PAH in animals who do not present measurable jets (Serres et al. 2007).

The indirect imagining markers measured and calculated in this study were not indicative of PAH. All AT values were higher than 64 ms, a threshold previously proposed to exclude a PAH condition (Shober and Baade 2006). The same authors suggested that AT/ET ratio values $\leq$0.25 were 100% specific for the diagnosis of PAH, while values higher than 0.42 excluded PAH. In this study, the calculated AT/ET ratio values ranged from 0.36 to 0.52, which were not indicative of PAH, as PAH could be excluded in six dogs. Two of the remaining four dogs with intermediate values were the same dogs that had mitral valve degenerative disease.

Vonk et al. (2007) indicated that Tei-index values higher than 0.36 are predictive of PAH, while values ranging from 0.28 to 0.36 are only indicative. In the absence of mitral valve disease, a high Tei-index value (0.48) was registered in only one

dog. No alterations of the other indices were registered in the same dog; thus, it was impossibile to combine additional indices to confirm the presence of PAH.

In conclusion, the preliminary results of this study do not support the presence of PAH in CanL. Nevertheless, as the pulmonary lesions registered in CanL make PAH a possible event, studies are in progress on an enlarged population to achieve more conclusive data.

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Chapter 21
Serum Amyloid A, Fibrinogen, and Haptoglobin as Inflammation Markers in the Horse: Preliminary Results

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Abstract The early detection and monitoring of inflammation is a primary challenge in veterinary medicine. The circulating concentrations of acute-phase proteins may provide an objective measure of both the severity of insult and the intensity of inflammatory responses in the horse. A clinical study on the possible role of serum amyloid A (SAA), fibrinogen, and haptoglobin as inflammation markers in the horse was conducted in 40 horses, with a significant correlation emerging between the patterns of SAA concentrations and clinical assessments of the patients being monitored during their hospitalization. The results of this preliminary study support the use of SAA as an important parameter at diagnostic, prognostic, and therapeutic stages. The sequential levels of SAA appear to be particularly useful in the postintervention monitoring of surgical patients.

Keywords Acute phase proteins • Equine • Fibrinogen • Serum amyloid A

21.1 Introduction

The immediate detection of an inflammatory response and the monitoring of its clinical course are primary challenges for veterinary medicine (Crisman et al. 2008). The search for early inflammation markers has therefore been an important focus of both veterinary and human medical research. Special attention has been on

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the identification of biochemical parameters that have the specificity and sensitivity to both signal the presence and evaluate the intensity of an inflammatory response (Jacobsen et al. 2005).

The acute phase reaction is a dynamic process that involves systemic metabolic changes that activate nonspecific defense mechanisms against any pathogenic *noxa* before specific defense responses are established. Such a highly organized physiological reaction involves changes in concentrations of plasma proteins termed "acute phase proteins" (Petersen et al. 2004). The hypothesis at the core of this research is that the circulating concentrations of these plasma proteins can provide an objective measure of both the severity of tissue injury and the intensity of the inflammatory response in horses, offering a useful contribution at both the diagnostic and therapeutic stages. Therefore, the objective of this preliminary research is to assess the role of serum amyloid A (SAA), fibrinogen, and haptoglobin as equine inflammation markers and biochemical parameters for the monitoring of the clinical course of horses that are affected by various pathologies.

21.2 Materials and Methods

This study was comprised of 40 horses (24 mares, 10 stallions, and six geldings; 31 adults and nine foals) that were hospitalized in the Department of Veterinary Pathology, Diagnostic and Clinic of the University of Perugia between April and June 2009. Twenty-one horses underwent surgery, 17 were treated medically, and consent for surgery was not obtained for the remaining two cases. Thirty-six horses were dismissed by the clinic and four were euthanized. The main reasons for referral were colic (19 adult horses), neonatal syndromes (eight foals), and noninflammatory orthopedic diseases (five horses). Thirty-nine horses were subjected to blood sampling upon admission. A subgroup of 24 horses was then selected for sequential blood sampling every 48 h (every 24 h in foals that were suspected to be affected by neonatal septicemia). Concentrations of SAA, fibrinogen, and haptoglobin were measured using a commercially available spectrophotometer that applies a latex-enhanced immunoturbidometric method for SAA and an immunoturbidometric method for the other two proteins (*Equinostic EVA1*).

21.3 Results

Upon admission, 28 horses had SAA plasma concentrations within the normal interval (0–20 mg/l) with values between 0 and 18.67 mg/l. The average value of this subgroup was 5.98 mg/l. Higher concentrations were measured in 11 patients, including five of the eight neonatal foals. A diagnosis of septic omphalophlebitis was formulated for the three foals with the highest SAA concentrations (ranging from 345.56 to 422.73 mg/l). Two of these cases were complicated by septic polyarthritis

and physitis, both of which led to unfortunate outcomes. The highest SAA concentration upon admission was found in a mare affected by bacterial hepatitis (546.58 mg/l). Fibrinogen concentrations upon admission were abnormal in 16 horses, though levels were only mildly elevated in four horses. Haptoglobin levels were measured in 19 horses, and only three neonatal foals had levels higher than the physiological limit.

Clinical monitoring through sequential blood sampling was implemented for 24 horses. Patients that underwent urgent laparotomy for noninflammatory pathologies featured low SAA concentrations upon admission, with one exception. SAA concentrations peaked 48 h after surgery and descended until they were normalized within 7 days. With the exception of a horse with a SAA peak of 41.39 mg/l, all other uncomplicated cases featured 48-h postsurgery SAA peaks between 234.18 and 463.72 mg/l. Horses with postsurgical complications showed either further elevation of SAA values with peaks higher that 600 mg/l (824.23 mg/l in one patient) or a concentration plateau beyond 400 mg/l. Horses that were admitted for elective surgery with less invasive procedures than laparotomy featured a similar SAA surgery response curve though with a lower 48-h peak (61.11 mg/l for arthroscopy and 177.59 mg/l for laryngoplasty and ventriculectomy). In all these patients, SAA concentrations returned to normal levels within 4 days after surgery.

21.4 Discussion

The main element of clinical interest is the significant correlation between SAA concentrations and the clinical assessments of the horses that were monitored during hospitalization. Therefore, the SAA surgery response scheme could provide, both for elective and urgent procedures, a useful clinical tool for postsurgical evaluation and early detection of complications, with special reference to septic cases. All uncomplicated cases featured a 48-h postsurgery SAA peak that promptly receded in the following days. A postpeak SAA plateau or further increases in SAA were always associated with the emergence of postsurgical complications. Hence, it would be appropriate to introduce the SAA sequential measurement in postsurgery monitoring protocols for both intensive-care patients and elective procedures. It is also interesting to note that, despite the limited sample size, the postsurgery peak was proportional to the severity of the surgical insult and the invasiveness of the procedure: minimal peaks following arthroscopy, medium peaks following laryngoplasty and ventriculectomy, and maximal peaks following laparotomy. Laparotomy wound infections that affected many patients did not alter the postsurgery SAA response curve, possibly because of its exclusive localization to the external suture planes and the limited systemic influence.

Postsurgery monitoring of fibrinogen and haptoglobin concentrations was not equally rewarding. While fibrinogen concentrations were often found beyond the normal range, no clear correlation was identified with the clinical assessment of the respective patients. Two main hypotheses were formulated to justify this evidence. First, fibrinogen concentrations can be altered by changes in hematocrit values and

modifications in vascular permeability, both of which are often present in postlaparotomy patients subjected to intensive fluid therapy. Second, plasma fibrinogen levels are influenced by its marked utilization in postsurgical coagulation and healing processes. Therefore, the use of fibrinogen as an acute-phase protein in postsurgical assessment is questionable. Similar considerations can be assumed for haptoglobin, as despite its oscillations, haptoglobin levels were often measured within the supposed normal range.

Monitoring of the medically treated horses also confirmed the strong correlation between plasma SAA concentrations, clinical assessments, and the extent of the response to therapy.

The limited sample size of this study does not allow for an adequate evaluation of the SAA contribution to diagnostic and prognostic formulations. However, admission data unequivocally confirmed the low physiological expression of SAA and the normality of the SAA plasma concentrations either in the absence of inflammation or within 4–8 h after onset. Therefore, anamnestic information on the suspected onset is of fundamental importance for the proper interpretation of SAA concentrations. Extremely elevated SAA plasma levels were also unequivocally detected upon admission in patients with septic conditions such as omphalitis and bacterial hepatitis. While the statistical significance of this evidence is hampered by the limited sample size, it is consistent with the reported high specificity of significant SAA elevations for septic cases.

The results of this preliminary study support the clinical use of SAA as a relevant biochemical parameter at both the diagnostic and prognostic stages, and for monitoring therapeutic responses. With the exception of hyper-acute cases, it is reasonable to exclude severe pathological conditions in patients with low plasma SAA concentrations. On the other hand, high plasma SAA concentrations can be considered unequivocal evidence of organic disease, due to either primary inflammation or noninflammatory conditions that induce secondary systemic consequences. In particular, extremely high SAA concentrations tended to be associated with bacterial infections. Responses to therapy were confirmed by the prompt reduction in plasma SAA levels. The absence of a decrease in SAA levels in response to therapies should promptly stimulate further diagnostic investigations and lead to the exploration of alternative therapeutic choices.

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Chapter 22
Comparison of a Semiautomated Electrophoretic System and a Quantitative Biochemical Method for the Evaluation of Alkaline Phosphatase Isoforms in Canine Serum

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Abstract In the dog, total alkaline phosphatase (TALP) activity represents the sum activity of isoforms produced by the posttranslational modification of two isoenzymes. In canine serum, TALP activity is primarily due to bone (BALP), hepatic (LALP), and corticosteroid-induced (CALP) isoforms. Different techniques are commercially available to measure ALP isoforms. The purpose of this study was to compare agreement of a semiautomated electrophoretic system (Hydragel 15 Iso-Pal) with a quantitative biochemical reference method that uses wheat-germ lectin precipitation and heat inactivation techniques. Concentrations of TALP, as well as BALP, LALP, and CALP isoforms, in 40 canine samples – including healthy dogs and dogs with hyperadrenocorticism – were determined using both methods. The Hydragel kit demonstrated low within-run and between-run precision when compared to the reference biochemical method and was unable to determine, with precision, CALP and BALP concentrations in dogs with hyperadrenocorticism.

Keywords Alkaline phosphatase • Dog • Electrophoresis • Isoforms

22.1 Introduction

Alkaline phosphatase (ALP) is an enzyme comprised of two isoenzymes in domestic mammals: intestinal and tissue-nonspecific isoenzymes. Posttranslational modification of the isoenzymes results in the creation of several isoforms, including bone (BALP), hepatic (LALP), and corticosteroid-induced (CALP) isoforms. Serum total ALP (TALP) activity is the sum activity of all circulating isoforms. In the dog, TALP activity is primarily due to BALP, LALP, and CALP isoforms

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(Hoffmann and Dorner 1975), with BALP constituting the largest component of TALP activity in young healthy dogs and LALP as the largest component in adult and old dogs (Syakalima et al. 1997a).

LALP can increase due to cholestasis caused by hepatic or extra-hepatic disease or due to the administration of certain medications (Gaskill et al. 2004). Serum CALP activity is seldom detected in young or adult healthy dogs but may be found in older dogs (Syakalima et al. 1998). Increases in CALP occur primarily from elevated serum cortisol levels induced by hyperadrenocorticism or iatrogenic causes but may also occur with diabetes mellitus, hypothyroidism, hepatobiliary disease, and chronic stress (Hoffmann et al. 1988). Several methods have been proposed to differentiate the isoforms of canine ALP; however, there is no gold standard. Biochemical methods, such as levamisole inhibition, heat inactivation (Teske et al. 1986), and selective precipitation with lectins (Syakalima et al. 1997a), provide adequate separation and quantification of canine ALP isoforms (Mahaffey and Lago 1991). Electrophoretic methods are also used, employing various support media, including cellulose acetate, agarose, and polyacrylamide. Samples may or may not be pretreated with *Vibrio cholerae* neuraminidase, and affinity techniques may be used, incorporating wheat-germ lectin (WGL) into the migration gel (Chamberlain et al. 1992; Bottoni et al. 2003). The aim of this study was to compare canine serum ALP isoform measurements from a commercially available electrophoresis kit [Hydragel 15 Iso-Pal (Sebia®)] with those from a biochemical reference method that utilized WGL precipitation and heat inactivation.

22.2 Materials and Methods

Nonfasting blood samples obtained from the cephalic vein of 40 dogs referred to the University of Milan were placed in vacuum tubes without anticoagulant. With the owner's written consent, surplus serum submitted for routine analysis was used. Twenty-four male and 16 female dogs of various breeds, ranging from 6 months to 13 years of age, were used in the study. Dogs were divided into three groups: group A – clinically normal dogs less than or equal to 1 year of age ($n = 10$); group B – clinically normal dogs between 1 and less than or equal to 7 years of age ($n = 15$); group C – dogs with untreated spontaneous hyperadrenocorticism ($n = 15$). Patient health status was determined by a full physical examination, complete blood count, and serum biochemistry profile. Spontaneous hyperadrenocorticism was diagnosed according to the literature (Feldman and Nelson 2004).

Upon collection, samples were immediately centrifuged at 3,000 $\times$ *g* for 5 min, and the serum, divided into two aliquots, was stored at -20°C for up to 2 weeks until analysis. TALP and ALP isoforms were measured immediately after thawing using electrophoretic and biochemical methods (1 aliquot/method). ALP isoform profiles were determined by a semiautomated agarose gel electrophoresis system (Hydragel Sebia®PN 1210, Issy-les-Molineux, France) not previously used in veterinary medicine, according to the manufacturer's instructions. Each aliquot

was divided, with half pretreated with wheat-germ agglutinin (WGA) to precipitate BALP, aiding identification of the LALP fraction. Both unadulterated and pretreated samples were electrophoresed concurrently in adjacent wells. The electrophoretic curves were generated using computer software (Phoresis, Sebia, Issy-les-Molineaux, France), with relative ALP isoform concentrations calculated as the percentage of optical adsorbance of the absolute ALP concentration (IU/L). A chemical autoanalyzer previously determined appropriate for veterinary use (COBAS MIRAS-Roche, Basel, Switzerland) was used as a biochemical reference method to measure TALP (Syakalima et al. 1997b). CALP and BALP were measured by heat inactivation and WGL precipitation (Sigma Chemical Co., St. Louis, MO, USA), respectively, as described in the literature (Teske et al. 1986; Syakalima et al. 1997b). LALP concentration was calculated by subtraction of BALP and CALP from TALP (Syakalima et al. 1998). Within-run and between-run precisions were calculated for all ALP isoform measurements obtained by both biochemical and electrophoretic methods [coefficient of variation (CV) = SD/mean × 100].

To establish within-run precision, two samples containing high (449 U/L) and low (98 U/L) ALP concentrations were analyzed with both biochemical and electrophoretic methods 10 times on the same day. Duplicate ALP isoform measurements for the same high and low ALP samples were obtained by both biochemical and electrophoretic methods, using the same batch of gels, on 5 different days during a 1-week period to estimate between-run precision. To assess linearity, one sample containing a high ALP concentration (2,400 U/L) was serially diluted 1:1, 1:2, 1:8, 1:16, and 1:32, and ALP isoforms were determined with both methods on every dilution. Linearity for each isoform was determined by linear regression of the two test methods. ALP isoform measurements obtained by the two methods were compared using Kruskal–Wallis tests ($P < 0.05$). Agreement between the two methods was analyzed using linear and Deming regression methods to assess correlation (R^2 values), and the presence of constant (intercept $\neq 0$) or proportional (slope $\neq 1$) errors using a Bland–Altman plot with bias was defined as the mean difference between methods. Statistical analyses were performed in MedCalc for Windows, version 9.2.1 (MedCalc Software, Mariakerke, Belgium).

22.3 Results

Within- and between-run precisions and linearities of the two test methods are presented in Table 22.1. The Hydragel method demonstrated separate migration patterns for unadulterated and pretreated samples only for groups A and B: unclear migration pattern divisions, with more fractions than expected occurred in group C. BALP and LALP concentrations were not significantly different between the two test methods in groups A and B (group A: $P_{BALP} = 0.701$, $P_{LALP} = 0.107$; group B: $P_{BALP} = 0.915$, $P_{LALP} = 0.428$). In group C, BALP concentrations were

Table 22.1 Within- and between-run precisions and linearities of the Hydragel and biochemical methods

Test/isoform	Within-run precision (CV)	Between-run precision (CV)	Linearity (R^2)
Hydragel method			
BALP	7.2	8.0	0.99
LALP	9.6	10.8	0.97
CALP	3.7	3.7	0.96
Biochemical method			
BALP	4.2	4.2	0.99
LALP	4.8	3.9	0.99
CALP	1.1	1.0	0.99

Table 22.2 Results of simple linear regression models fit to measurements of ALP isoforms determined by Hydragel and biochemical methods

Isoform	Slope (95% CI)	Intercept (95% CI)	R^2	Error
BALP	0.98 (0.95–1.02)	−4.91 (−10.59–0.76)	0.99	No
LALP	1.21 (0.54–1.87)	−11.57 (−56.01–32.87)	0.36	No
CALP	0.39 (−0.04–0.82)	60.05 (8.89–111.22)	0.27	Constant and proportional
BALP (group C)	−0.38 (−0.80–0.04)	17.93 (4.59–31.26)	0.22	Constant and proportional

significantly different ($P = 0.005$) between the two test methods. The CALP isoform was only found in group C.

Simple linear regression models fit to the data demonstrated good correlation (R^2 close to 1) only for BALP (Table 22.2). For the CALP isoform, constant and proportional errors were present. These findings were supported by a Bland–Altman plot (mean bias = 22.6). Deming regression analysis also demonstrated constant and proportional errors for BALP in group C.

22.4 Discussion

The Hydragel system was easy and fast to operate, providing standardized analyses and diminished procedural errors. Overall, correlation between the Hydragel and COBAS methods was low. When all 40 dogs were included, BALP measurements for the two methods were highly correlated; but if only data from group C were analyzed, the correlation was poor, and constant and proportional errors were present. The Hydragel system was capable of obtaining good electrophoretic patterns only for healthy dogs. Electrophoretic migrations of dogs with hyperadrenocorticism had incorrect subdivisions, causing constant and proportional errors for CALP and BALP isoform measurements. The Hydragel system has good reproducibility, precision, and specificity when analyzing human serum (Van Hoof et al. 1993). The main difference between human and canine serum ALP is the presence

of CALP in canine serum. For this reason, we suppose that the CALP isoform was the cause of the incorrect subdivision in some electrophoretic patterns. CALP could create interference with the migration of other isoforms, in particular, BALP. The Hydragel system is only suitable to measure ALP isoforms in healthy dogs and, therefore, not applicable in canine clinical practice. It may be interesting to modify the voltage or contact times between the serum and gel during the automatic phase of the Hydragel procedure, using a greater number of samples with CALP, to see whether it is possible to improve the performance of the system.

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Chapter 23
Canine Inflammatory Bowel Diseases: Diagnostic and Therapeutic Considerations on 19 Cases

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Abstract The aim of the present study was to evaluate diagnostic and therapeutic features and to search correlations between clinical, endoscopic, and histological scoring in 19 dogs with chronic inflammatory bowel disease. Additionally, these parameters were evaluated in dogs with normo-albuminemia versus hypoalbuminemic dogs. Therapy and 6-month follow-up data were reported. No differences were found between clinical, endoscopic, and histological scores in normo-albuminemic and hypoalbuminemic dogs. The cornerstone of therapy was prednisone associated with metronidazole. In subjects unresponsive to steroids, treatment with other immunosuppressive drugs must be considered.

Keywords Dog • Hypoalbuminemia • IBD • Therapy

23.1 Introduction

The term inflammatory bowel disease (IBD) is applied in veterinary medicine to identify a group of idiopathic disorders characterized by histopathological evidence of inflammation in the lamina propria of the small or large intestine. The aims of this study were to, in dogs affected with IBD, (a) describe the clinical aspect, treatment, and long-term outcome; (b) correlate the scores of clinical signs with endoscopic and histological evaluation; and (c) evaluate these parameters in hypoalbuminemic versus normo-albuminemic dogs with inflammation of the small intestinal tract.

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23.2 Material and Methods

Records of dogs in which IBD had been diagnosed at the Unit of Medicine and Pharmacology, University of Messina (Italy) were retrospectively reviewed. Criteria for inclusion in the study were persistent gastrointestinal clinical signs (>3 weeks in duration), failure to respond to antibiotics and/or dietary therapies carried out for at least 3 weeks, histopathological evidence of gastric, small intestinal, or colonic inflammation, and failure to document other causes of symptoms by thorough diagnostic evaluation (exocrine pancreatic insufficiency, endoparasites, neoplasia, etc.). Calculation of the clinical assessment of disease activity by CCECAI was scored 1–12. Hematological tests, serum biochemical profiles (with folate, cobalamine, and trypsin-like immunoreactivity measurements), and urinalysis were performed for all dogs. Repeated fecal flotation examination was negative for parasitic ova and *Giardia* cysts. Endoscopy of the affected gastrointestinal tract was performed. Multiple endoscopic biopsies were taken from the gastrointestinal tract explored. The lesions revealed during endoscopic examination were classified as normal mucosa [0]; slightly friable and edematous mucosa [1]; friable mucosa with superficial, whitish specks [2]; and friable mucosa easily bleeding and poorly relaxing insufflation [3] (Jergens et al. 1992). The histopathological evaluation of biopsies, sectioned at 3–4 μm and stained with EE and PA, performed blind by a single veterinary pathologist, was based on architecture of tissues and distribution of inflammatory cells. Severity of changes was scored on a scale from 0 (normal), 1 (mild – inflammatory infiltration within changes of architecture or mucosal epithelial immaturity), 2 (moderate – mucosal epithelial immaturity and/or necrosis alone), or 3 (severe – inflammatory infiltration with multifocal necrosis of epithelium or massive architectural distortion) (Washabau et al. 2010).

All dogs were fed a new protein commercial diet and had been given intermittent antibiotic treatment with either transient or no clinical improvement. A monthly follow-up was carried out for 6 months after the end of pharmacological treatment by assessing the presence of clinical signs and the dietary management adopted. Disease outcome at the time of follow-up was described as either "remission," "intermittent signs," or "uncontrolled disease." Dogs in remission were defined as those free of clinical signs for the preceding 6 months. Those with "intermittent clinical signs" were defined as experiencing clinical signs more frequently than every 14 days. Dogs with "uncontrolled disease" were defined the subjects symptomatic during the therapy. The Spearrman test was used to evaluate the correlation between CCECAI, endoscopic scores, and histopathological scores. The Mann–Whitney test was used to compare the CCECAI with endoscopic and histopathological scores in hypoalbuminemic versus normoalbuminemic dogs with inflammation of the small intestine. Statistical significance was associated with a P value less than 0.05.

23.3 Results

The median age of dogs was 5.2 years (range: 4 months–14 years) for nine males and 10 females. Sixteen dogs were purebreds; three were mixed breed. The median duration of clinical signs prior to diagnosis was 7.7 weeks (range: 3–12 weeks). The median score of CCECAI was 6.8. Five dogs were affected by IBD classified as mild (score 4–5), nine by moderate (6–8), four by severe (9–11), and one by very serious IBD ($\geq$12). Hematological abnormalities were present in 10/19 dogs (52.6%), including regenerative anemia in two (10.5%; media PCV = 19%, reticulocytes = 8.2%), leukocytosis in seven (36.8%), neutrophilia (media 20.78 cells/μl) in six (31.5%), neutropenia (media 2.8 cells/μl) in two (10.5%), thrombocytosis (media 570 cells/μl) in two (10.5%), and thrombocytopenia (media 145 cells/μl) in three (15.7%). Serum biochemical abnormalities were observed in 13/19 cases (68.4%). Alanine aminotransferase was elevated in eight dogs (42.1%), alkaline phosphatase in three (15.7%), and gamma glutamyl transferase in one (5.2%). Hypotriglyceridemia was present in three subjects (15.7%), hypocholesterolemia in two (10.5%), hypoalbuminemia (range 0.9–1.5 g/dl; v.n. 2.5–4.4 g/dl) in six (31.5%). Folate levels were elevated in one dog (32 ng/ml; v.n. 7–17 ng/ml); in two dogs, folate and cobalamine levels were decreased (mean 135 pg/ml; range: 225–860 pg/ml). The endoscopic examination revealed mucosal lesions in 14 dogs. The endoscopic mean score was 6; two dogs showed mild lesions, six moderate, and six severe. The diagnosis was lymphocytic duodenitis in 14 dogs, lymphocytic colitis in two, histiocytic ulcerative colitis in three, and eosinophilic in two. In only one case of lymphocytic enteritis, a secondary lymphangectasia, with an endoscopic mean score of 1.8, was observed. The CCECAI index was significantly related with the endoscopic score ($P = 0.012$). No connection was found between CCECAI and histopathological score ($P = 0.20$). Endoscopic and histopathological scores were very significant ($P = 0.007$). No significant scores of clinical signs were found between hypoalbuminemic and normoalbuminemic dogs with disorders of the small intestine ($P = 0.11$); in those animals, no connection was present between endoscopic ($P = 0.10$) and histopathological ($P = 0.22$) assessment. Treatment with prednisone (1–2 mg/kg/BID for 2–3 months at dose scale) was performed in 18/19 dogs. In all cases, it was associated with metronidazole (20–30 mg/kg/BID) and, in dogs with the colon involved, with enrofloxacin (5 mg/kg/SID) and sulfasalazine (10–20 mg/BID). In one case, with serious hypoalbuminemia, lymphangectasia, and failure response at steroid treatment, cyclosporine (5 mg/kg/SID orally) was given for 45 days. Following failure response to cyclosporine, methotrexate (0.6 mg/kg/week) was given by i.m. in three administrations. In one dog with histiocytic ulcerative colitis (HUC) and ehrlichiosis, treatment with doxycycline (10 mg/kg/SID for 30 days) was carried out with prednisone and sulfasalazine; clinical signs fully resolved but had only partially been controlled previously. A low concentration of serum albumin was found in dogs with a slower disappearance of clinical signs and, therefore, these animals needed a longer treatment period.

In two dogs with hypocobalamine, cobalamine was administered by i.m. at a dose of 500–1,000 μg every 7 days until normalization of values. During follow-up, two dogs died of extraintestinal causes, and two others without hypoalbuminemia were euthanized at the request of the owners.

23.4 Discussion

The findings reported in this paper are in accordance with other authors (Jergens et al. 1992; German et al. 2003). There were no effects of sex, breed, or age predisposition on the findings. It is interesting to note that HUC has been detected in two breeds not previously reported (West Highland White Terrier and Cavalier King Charles Spaniel), and 77.2% of subjects belonged to pure breeds: four were German shepherds, two of which had the same father. Regenerative anemia, leukocytosis with neutrophilia, and the presence of band neutrophils were observed in subjects at a visit after a long period from the onset of symptoms. Thrombocytosis observed in two dogs is a common extraintestinal manifestation of IBD in humans. In human studies, thrombocytosis is proportional to the activity of the disease and is related to reduced half-life and increased platelet turnover (Talstad et al. 1973). In our cases, in dogs affected with lymphocytic enteritis presenting on histopathology, clinical evaluation scores were higher. Thrombocytopenia is considered a random finding in human and canine IBD, although its onset may be immunological (Ridgway et al. 2001). No significant differences between various assessment criteria of disease (CCECAI, endoscopy, and histopathological examination) were seen in hypoalbuminemic and normoalbuminemic dogs, although we examined a small number of subjects, indicating that the laboratory findings did not relate to the severity of clinical, endoscopic, and histological findings. Additionally, if the endoscopic examination of lymphangiectasia is indicative of protein-loss enteritis, the severity of endoscopic images may not correlate with hypoproteinemia. There was a significant correlation between CCECAI and endoscopic score in accordance with a recent study in which the subjects with small intestine disease showed prognosis from guarded to unfavorable (Allenspach et al. 2006). The relationship between endoscopic and histopathological scores was significant in our cases but was not always detectable because of variations in the subjective assessment (endoscopist operator vs. histopathologist) and technical quality of the biopsies. In contrast with Allenspach (2007) where hypocobalamine was considered an indicator of negative response to immunosuppressive therapy, our dogs with low levels of cobalamine responded to treatment, albeit with longer times. High ALT levels may be associated with subclinical disease of the hepatobiliary system, characterized histologically by mononuclear infiltrates in periportal vasculature and involvement of the liver with upward departure from the gut (Marks 1998).

Treatments for IBD involve a combination of pharmacologic and nutritional therapies. For mild forms, management may be only dietary with the use of *novel protein*. In moderate or severe forms, dietary management should be combined with

pharmacologic treatment. Corticosteroids represent the treatment of choice for their immunosuppressive properties. They have often been combined with azathioprine or metronidazole for more aggressive treatment and to reduce the dosage needed (Saxena et al. 1985). Enrofloxacin was effective for the treatment of HUC and is efficacious against infectious agents, which, as recently demonstrated, appear to be responsible for the disease (Davies et al. 2004). In one case of HUC, excellent efficacy was inadvertently obtained with doxycycline. Doxycycline beyond antimicrobial activity inhibits the synthesis of nitric oxide found in high quantities in the colon of subjects with chronic colitis (Amin et al. 1996). Methotrexate, an antimetabolite of folic acid, has immunosuppressant and anti-inflammatory activities and has been effectively used in a dog with protein-loss enteropathies and lymphocytic enteritis that were unresponsive to steroids and cyclosporine (Yuki et al. 2006). These results allow consideration of possible therapeutic choices in the treatment of IBD (Allenspach et al. 2007). In conclusion, despite the difficulties in treating IBD, the use of different therapeutic protocols and laboratory findings allows good results to be obtained.

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Chapter 24
Influence of Abdominal Surgery on Pulmonary Atelectasis Formation in Dogs

F. Staffieri, V. DeMonte, C. DeMarzo, S. Grasso, and A. Crovace

Abstract The aim of this study was to evaluate the influence of abdominal surgery on atelectasis formation in healthy dogs. After the induction of general anesthesia (GA), 20 dogs, scheduled for elective ovariohysterectomy, were positioned in dorsal recumbency: 10 dogs underwent immediate surgery (S group), while 10 dogs (NS group) were maintained under anesthesia for 60 min before surgery. In both groups, a helical computed tomography (CT) scan of the thorax and an arterial blood gas analysis were performed 60 min after the induction of GA. Lung aeration was estimated by analyzing the radiographic attenuation of the CT images. The atelectasic and poorly aerated lung compartments were significantly larger, and the normally aerated lung compartment was smaller in the S group compared to the NS group. The PaO_2 was similar in both groups. Abdominal surgery significantly increases pulmonary atelectasis in healthy dogs under GA.

Keywords Dog • General anesthesia • Pulmonary atelectasis • Surgery

24.1 Introduction

Pulmonary atelectasis is one of the major factors responsible for the derangement of gas exchange during general anesthesia (Duggan and Kavanag 2005). The mechanisms responsible for pulmonary atelectasis formation during general anesthesia are (1) compression of the pulmonary parenchyma (compression atelectasis), (2) absorption of the alveolar gas (absorption atelectasis), and (3) alteration of the surfactant function (Duggan and Kavanag 2005). All these factors contribute simultaneously and independently to the formation of atelectasis. Abdominal

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surgery is usually associated with an increased intra-abdominal pressure that interferes with the diaphragmatic function, promoting alveolar collapse (Lundh and Hedenstierna 1983). The aim of this study was to evaluate the influence of the abdominal surgery on pulmonary atelectasis formation in healthy dogs undergoing ovariohysterectomy.

24.2 Materials and Methods

Twenty healthy female dogs scheduled for elective ovariohysterectomy were included in this study. Dogs were premedicated with acepromazine (30 μg/kg) and morphine (0.3 mg/kg) administered intramuscularly (IM). General anesthesia was induced with intravenous (IV) thiopental (10 mg/kg) and maintained with inhalation of isoflurane in 100% oxygen. After intubation, all patients were mechanically ventilated (Aliseo, Datex Ohmeda, Helsinki). During premedication, induction, and intubation, all dogs were restrained in sternal recumbency, and when an adequate level of anesthesia was achieved, dogs were positioned in dorsal recumbency and maintained in this position until the end of the study. Heart rate (HR beats/minute) systolic, mean, and diastolic noninvasive arterial pressures (mm Hg), respiratory rate (RR, breaths/minute), hemoglobin oxygen saturation (SpO_2, %), and the end-tidal partial pressure of carbon dioxide ($EtCO_2$, mmHg) were continuously monitored and recorded every 5 min during the study. All dogs were ventilated in a volume-controlled mode with a tidal volume (V_T) of 12 ml/kg, an inspiratory to expiratory ratio (I:E) of 1:2, a positive end expiratory pressure (PEEP) of 0, and an inspired oxygen fraction (FiO_2) greater than 0.9. Randomly, 10 dogs underwent surgery immediately after the stabilization of the general anesthesia (S Group), and the other 10 dogs underwent surgery after the execution of the thoracic computed tomography (CT; NS Group). In all dogs, 60 min after positioning in dorsal recumbency, a thoracic spiral CT was performed, and an arterial blood sample was collected. Dogs of the S group were excluded from the study if, after 60 min from positioning in dorsal recumbency, they were still undergoing surgery. For the execution of the thoracic CT, each dog was positioned in the scanner in dorsal recumbency, and a dorsal plane scout image was obtained. Spiral CT of the thorax was then performed during end-expiration apnea. All images were obtained at a setting of 120 kVp and 160 mA using a lung algorithm; matrix size was 512 × 512, field of view was 35, and pitch was 1.5. Images of 10-mm slice thickness were reconstructed. All CT images were analyzed for lung abnormalities; if pathologic changes were observed, the dog was excluded from the study. Lung densities were analyzed in all CT images with specific software (Dicom Works v 1.2.5) as previously described (Staffieri et al. 2007, 2009, 2010). The total area (mm^2) of the entire lung was calculated by including pixels with density values between −1,000 and +100 Hounsfield units (HUs). In accordance with previous studies, the following lung compartments were identified: hyperinflated (−1,000 to −901 HUs), normally aerated (−900 to −501 HUs), poorly

aerated (−500 to −101 HUs), and nonaerated (atelectasis; –100 to +100). The total area that each compartment occupied within the entire lung was computed and then expressed as the percentage of the entire lung area. Gas exchange was evaluated for each patient by measuring the arterial partial pressure of oxygen (PaO_2) and carbon dioxide ($PaCO_2$). All data are expressed as mean ± standard deviation, and normal distribution was tested with the Kolmogorow–Smirnow test. Data related to gas exchange and pulmonary aeration (% of hyperinflated, normally aerated, poorly aerated, and nonaerated compartments) were compared between the two groups with the one-way analysis of variance (ANOVA) test ($P < 0.05$).

24.3 Results

The two groups were similar in terms of body weight (NS = 21.4 ± 4.2 kg; S = 20.6 ± 7.2 kg) and age (NS = 35.3 ± 6.3 months; S = 38.2 ± 6.3 months). The study was conducted without any complications, and none of the dogs were excluded. The median duration of surgery in the S group was 46.2 ± 7.7 min; thus, in all dogs of the group, it was possible to perform the CT study. Compared to the NS group, the S group had a significantly greater amount of nonaerated (atelectatic; NS = 3.9 ± 1.3%; S = 12.8 ± 3.7%) and poorly aerated (NS = 20.4 ± 7.2%; S = 26.7 ± 5.3%) lung compartments and a smaller amount of the normally aerated lung compartment (NS = 74.4 ± 7.8%; S = 58.9 ± 8.1%; Fig. 24.1). The percentage of hyperinflated lung compartment (NS = 1.1 ± 0.8%; S = 1.2 ± 0.8%), the mean value of PaO_2 (NS = 499.2 ± 65.3 mmHg; S = 491.2 ± 49.2 mm Hg), and $PaCO_2$ (NS = 38.3 ± 4.2 mmHg; S = 37.1 ± 3.5 mmHg) were similar in both groups.

24.4 Discussion

The main result of this study is that in healthy dogs under general anesthesia, abdominal surgery interferes with pulmonary aeration, promoting the formation of atelectatic and poorly aerated lung compartments and reducing the normally aerated lung area. We can speculate that the increased intra-abdominal pressure induced by the surgical procedures promotes the cranial displacement of the diaphragm with the consequent reduction of the pulmonary functional residual capacity, and consequently promotes airway closure and alveolar collapse (Duggan and Kavanag 2005; Hedenstierna 2003). Despite the greater impairment of lung aeration, arterial oxygenation was not significantly affected in dogs of the S group compared to dogs of the NS group. The apparently discordant results can be justified by two theories: the use of high FiO_2 compensated for the greater impairment of lung aeration, assuring similar arterial oxygenation in dogs of the S group; we can also speculate that dogs of the S group had a lower cardiac output as a result of the deeper

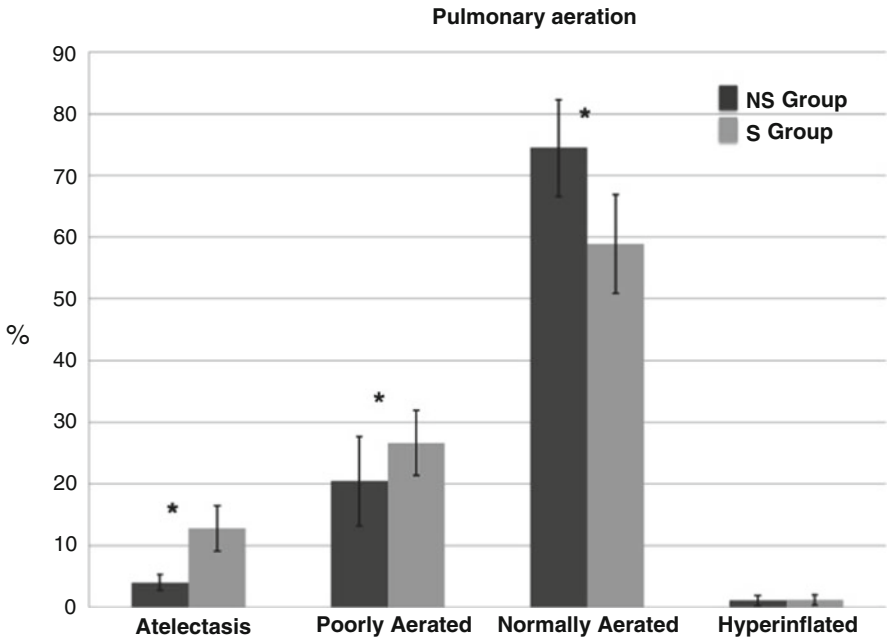

Fig. 24.1 Percentage of the lung compartments in 20 dogs under general anesthesia. The S group includes dogs ($n = 10$) that underwent surgery before the CT scan, the NS group include dogs ($n = 10$) that did not undergo surgery before the CT scan; *$P < 0.05$ (one-way ANOVA)

level of anesthesia, abdominal compression, water loss, and bleeding related to the surgical procedures. Several experimental and clinical studies demonstrated that cardiac output might interfere with the percentage of the intrapulmonary shunt. In particular, lower cardiac outputs, within certain limits, reduce the intrapulmonary shunt improving patient oxygenation (Lynch et al. 1979).

Pulmonary atelectasis not only interferes with respiratory mechanics and gas exchange but can also be associated with postoperative pulmonary complications characterized by the permanence of gas-exchange impairment and the development postoperative pulmonary infections (Duggan and Kavanag 2005). Based on the results of this study, abdominal surgery promotes pulmonary atelectasis formation, and thus, the use of PEEP and lung-recruitment maneuvres is recommended to preserve lung aeration during abdominal surgery (Staffieri et al. 2009, 2010).

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Part IV
Food Inspection

Chapter 25
Ice Fish (*Protosalanx* spp. and *Neosalanx* spp.) and Rare Fish Species (*Sardinia pilchardus* and *Aphia minuta*): microbiological Evaluation for Hygienic Health Assessment and Consumer Protection

C. Bacci, A. Paris, E. Boni, S. Bonardi, L. Bassi, I. Alpigiani, and F. Brindani

Abstract In recent decades, there has been a significant increase in consumer demand for fishery products from aquaculture or imported from foreign countries. In this study, microbiological parameters (*Listeria monocytogenes*, *Salmonella enterica*, *Vibrio paraheaemolyticus*, *Escherichia coli*, and enumeration of mesophilic and psychrotrophic microorganisms) regarding fish belonging to *Protosalanx* spp. and *Neosalanx* spp. (imported from China), *Sardina pilchardus* juveniles (also known as "bianchetto"), and *Aphia minuta* (called "rossetto") were evaluated. Imported fish often replace morphologically similar species with a greater commercial value.

Keywords *Aphia minuta* • Ice fish • Microbiological evaluation • *Sardina pilchardus*

25.1 Introduction

In recent years, a remarkable rise in the consumer demand for fishery products has been observed, resulting in an increase in imports and development of aquaculture techniques (Croci 2004). As indicated by the Border Inspection Posts, most of the fish exchanged in the international market come from developing countries, which often have inadequate food control systems and a high incidence of foodborne outbreaks. In such countries, fishing occurs in prohibited areas, and fishery product transport occurs in unreliably hygienic conditions, often without cold-chain maintenance (Toti 2004). Beside a regulatory framework, which often protects the consumer, the Inspectorate for Frauds Prevention and Control blames the alarming increase in complaints concerning food imported from Asiatic countries. The list of

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fraudulent and illegal products coming from China is extremely long: among them, the retail of imported ichthyic species (*Protosalanx* spp. and *Neosalanx* spp.), named "ice fish," for morphologically similar species with a greater commercial value, such as "bianchetto" (juvenile *S. pilchardus*) and "rossetto" (adult *A. minuta*), is recurring. Other factors that may considerably influence the quality of the product, which is perishable, are the scarce information on fishing areas (sweet and brackish water) and procedures, water microbial quality, freezing speed, transport duration, and cold-chain maintenance. It is therefore appropriate to perform a microbial characterization of these products, including qualitative pathogen identification (*Listeria monocytogenes*, *Salmonella enterica*, and *Vibrio parahaemolyticus*) and the determination of the extent of spoilage bacteria (mesophiles and psycrophiles) (Shin et al. 2004; Twedt 1989).

25.2 Materials and Methods

The microbial characterization of 43 samples of fresh fish (PF; 23 belonging to *S. pilchardus* and 20 to *A. minuta*), coming from the Adriatic, Tyrrhenian, and Ligurian seas, was carried out. Samples were found in supermarkets and fishery markets in the provinces of Parma and Pisa from 15 February to 15 April. Twenty-one samples of ice fish (PG) and relative defreezing waters (PG H_2O) from the sweet water of China and purchased at supermarkets in the above-mentioned provinces were investigated. Regarding the hygienic assessment, mesophilic aerobic count determinations of fresh fish (ISO standard 4833:2003) and psycrophilic aerobic count determination of thawed fish (ISO standard 17410:2001) and the defreezing waters were carried out. The presence of *Escherichia coli* (most probable number, MPN) (ISO standard 7251:2005) was also evaluated to verify potential fecal contamination, resulting from the fishing waters. Detection of the pathogens *S. enterica* and *L. monocytogenes* was carried out according to the standards UNI EN ISO 6579:2008 and UNI EN ISO 11290–1:2005 (Reg. EC 2073:2005). Isolation of *V. parahaemolyticus*, not yet considered in the Regulation, was determined according to the standard ISO/TS 21872–1:2007. The isolated *Salmonella* spp. strains were serologically and genotypically identified (by PCR-Simplex). Afterwards, phenotypic (Kirby Bauer method) and genotypic (PCR-Simplex) typing were executed to detect the presence of plasmid genes and virulence chromosomes. For this purpose, 17 antibiotics were tested: enrofloxacin (5 μg), ciproploxacin (5 μg), streptomycin (10 μg), colistin sulfate (10 μg), gentamicin (10 μg), cefotaxime (10 μg), sulfamethoxazole/trimethoprin (25 μg), nalidixic acid (30 μg), cephalothin (30 μg), kanamycin (30 μg), tetracycline (30 μg), neomycin (30 μg), chloramphenicol (30 μg), amoxicillin/clavulanic acid (30 μg), ampicillin (30 μg), ceftazidime (30 μg), and sulfonamide compounds (300 μg).

From each overnight culture, genetic material was extracted using a chelating resin (instaGene™ Matrix, BIORAD). Twenty to 24 μl of a Master Mix consisting of sterile, distilled water (Fermentas), Taq Buffer 1× (Fermentas), 0.2 mM of each

dNTP (Fermentas), primer (from 0.2 to 1 μM, Sigma Genosys), 3 U/50 μl AmpliTaq DNA polymerase (Fermentas), and 4 mM $MgCl_2$ (Fermentas) were added to the extracted DNA (from 1 to 5 μl).

A pair of primers, Flic-s/Flic-as (Lim et al. 2003), was used for the identification of the *flicC* gene in *S. typhimurium* serotypes. Pathogenic gene fragments were identified through amplification with the following pairs of primers: ST 139/ ST 141 (*inv*A gene, 284 bp), 337 L/338 R (*pho*P gene, 299 bp), SpvC for/SpvC rev (*spv*C gene, 699 bp), and SpvR 1/SpvR 2 (*spv*R gene, 891 bp) (Rhan et al. 1992; Way et al. 1993; Abouzeed et al. 2000; Haneda et al. 2001). Pulsed-field gel electrophoresis (100 V, 498 mA) was performed in a 2% agarose gel (Eppendorf) for 2 h.

25.3 Results

The values of the psycrophilic aerobic count in the 21 samples of ice fish ranged between 2.62 log CFU/g and 6.47 log CFU/g (mean value: 4.75 log CFU/g), while values in the relative defreezing waters fluctuated between 3.87 log CFU/ml and 7 log CFU/ml (mean value: 5.14 log CFU/ml). The mesophilic aerobic count in the fresh fish varied between 2.91 log CFU/g and 8.61 log CFU/g, with a mean value of 5.86 log CFU/g (Fig. 25.1). This last result depended on the geographic origin: 5.49 log CFU/g (Adriatic Sea), 6.12 log CFU/g (Tyrrhenian Sea), and 4.97 log CFU/g (Ligurian Sea). The MPN method highlighted *E. coli* contamination in 23.8% of the samples of ice fish, 14.3% of defreezing waters, and 7.0% of fresh fish. Two samples (PG 6 and PG 16) were negative using the MPN method, but *E. coli* was identified from selective media used in the isolation of *S. enterica* and *V. parahaemolyticus*. No samples were positive for *V. parahaemolyticus* and *L. monocytogenes*.

L. grayi was isolated from 8% of the samples, *L. ivanovii* from 3.5%, and both *L. innocua* and *L. welshimeri* from 2.3% of the samples. *S. napoli*, sensitive to all tested antibiotics, and *S. typhimurium*, resistant to streptomycin, ampicillin, chloramphenicol, and sulfonamide, were isolated, respectively, from two samples of ice fish (PG 15 and PG 21). For the first serotype, only genes *invA* and *phoP* were identified, while *S. typhimurium* possessed the *fliC* gene (Fig. 25.2), which

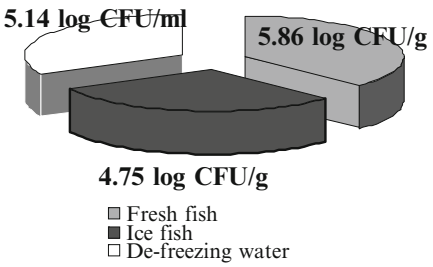
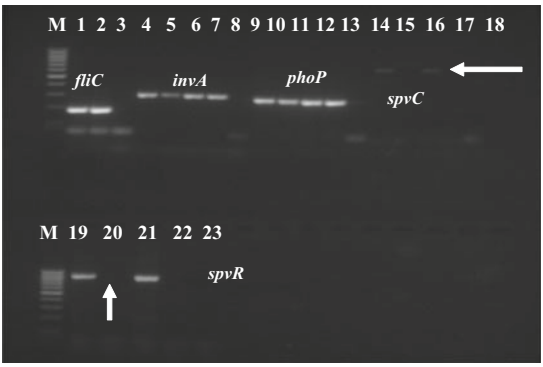


Fig. 25.1 Mean value of the mesophilic and psycrophilic aerobic counts of fresh fish, ice fish, and defreezing water



M: Mass Ruler™ DNA Ladder Low Range, (Fermentas)
Lanes 1, 6, 11, 16, 21: *S. typhimurium*; 2, 4, 9, 14, 19: *S. typhimurium* ATCC 14028; 3, 8, 13, 18, 23: negative control; 7, 12, 17, 22: *S. napoli*; 5, 10, 15, 20: wild-type strain *S. enteritidis*

Fig. 25.2 Pulsed-field gel electropherogram of the amplicons *fliC*, *invA*, *phoP*, *spvC*, and *spvR* in the isolates of *S. enterica*

identified the serotype, and the virulence factors examined. *Escherichia adecarboxylata, E. hermannii*, *Enterobacter cloacae*, and *Citrobacter youngae*, microorganisms widespread in the environment but not necessarily related to fecal contamination, were isolated from 19.0% of the frozen samples. *Shewanella putrefaciens*, a spoilage bacterium highly widespread in frozen and chilled fishery products, was isolated in PG H_2O 11.

25.4 Discussion

The values related to mesophilic aerobic counts for fresh products, recommended by the International Commission on Microbiological Specification for Foods (ICMSF), must be below 7 log CFU/g. Twenty percent of the samples of *A. minuta* and the 8.7% of the samples of *S. pilchardus* exceeded this limit. This finding could be due to the habitats where these species live: "rossetto" lives in sandy and muddy seabeds at a depth of 80 m, while "bianchetto" lives generally between 25 and 35 m (Relini et al. 1999; Lucchetti 2006).

The values related to psycrophilic aerobic count recommended by the Frozen Food Institute for frozen products are between 5 log CFU/g and 6 log CFU/g. In the survey, 14.3% of the samples, both ice fish and defreezing water, exceeded 6 log CFU/g. Considering that freezing causes a numerical reduction in the microorganisms initially present in the food, the obtained results show that these species could be fished in coastal waters, which are more polluted. The obtained results with the MPN method prove low hygienic levels, especially in frozen products, allegedly due to the deterioration of the defreezing water and subsequent handling.

This finding is confirmed by the presence of spoilage microflora, responsible for fish deterioration, and the presence of two different serotypes of *S. enterica*, one of which is pathogenic.

Acknowledgment Dr. Enrico Padovan is acknowledged for valuable and essential collaboration.

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Chapter 26
Study of the Resistance Variability of *Anisakis* Larvae to Some Technological Stressors

F. Giarratana, A. Giuffrida, F. Gallo, G. Ziino, and A. Panebianco

Abstract The aim of this work was to assess the resistance variability of a large number of *Anisakis* larvae ($n = 1{,}200$) to certain food technology stressors such as marinade and thermal treatment, with particular regard to the relationship between stress resistance and length (in millimeters). The larval inactivation rate for marinating treatment showed the highest resistance in the longest larvae when the concentration of NaCl and organic acids were higher, while for milder treatments, the resistance was inversely proportional to length. Concerning thermal treatments, the resistance observed was approximately, but directly, proportional to larvae length.

Keywords Anisakis larvae • Marinade • Thermal treatments • Resistance treatments • Seafood

26.1 Introduction

The presence of anisakid larvae in seafood products is an important food safety issue due to the large number of involved fish species, the epidemiological dynamism, the severity of the induced human disease, and the allergenic capacity of devitalized larvae (Audicana et al. 2002). For these reasons, the regulation (EU Regulation 853/2004) concerning the production of "fishery products to be consumed raw or almost raw," as well as "marinated and/or salted fishery products, if the processing is insufficient to destroy nematode larvae" establishes a freezing treatment of −20°C for 24 h. Since freezing can affect the sensorial characteristics of marinated or slightly salted fishery products, several alternative methods were studied to obtain an equivalent effect. Particularly, several studies (Adams et al. 1999;

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Arcangeli et al. 1996; Gianfaldoni et al. 1990; Priebe et al. 1973) assessed the efficacy of some salting and/or marinating technologies on larval inactivation rates, as well as on the reduction of their pathogenicity. However, results showed a high variability in parasite responses, which could be due to the degree of development of the larvae or their encystment status, both of which are related to the modality of fish infestation, the environmental conditions of the fishing site, the morphological and immune conditions of the gastroenteric tracts of the parasitized fish (Panebianco et al. 1999), and the redox potential of fish celomic cavity (Panebianco et al. 2000).

Apart from these causes, a careful knowledge of the variable anisakid larvae responses to the most common food technologies, such as salting, marinating, or mild thermal treatments, could be very useful for correctly assessing related food safety issues and the individuation of effective inactivation treatments. The aims of this work were to assess the variability of responses of a large number of anisakid larvae as a function of their length and to determine correlations applicable to predictive algorithms concerning larval inactivation.

26.2 Materials and Methods

The present study was performed on 1,200 motile anisakid larvae sampled from eight specimens of *Lepidopus caudatus* in a physiological solution that were harvested within 8 h of sampling. Ten percent of the larvae were examined microscopically to determine the genus. Afterward, 600 larvae were subdivided into three groups (200 larvae for each group) and treated with three different marinades: group A: 5% citric acid, 5% acetic acid, and 13% NaCl; group B: 5% citric acid, 5% acetic acid, and 10% NaCl; and group C: 2.5% citric acid, 2.5% acetic acid, and 8% NaCl. Larval survival was evaluated by stereomicroscopy every 3 h for group A, every 6 h for group B, and every 12 h for group C. All groups were maintained at 30°C during the study to increase the dissociation of organic acids to enhance their activity against the larvae.

The remaining 600 larvae were subjected to sublethal thermal treatments. Specifically, 400 larvae were preliminarily tested at 45, 47, 48, and 50°C to assess the temperature that resulted in 100% inactivation of the larval within an appropriate time interval. Based on this experiment, 200 larvae were treated at 47°C and were stereomicroscopically observed (devitalization assessment) after 15, 20, 25, 30, and 40 min.

All thermal treatments were performed by immersing larvae in glass test tubes containing 15 ml of prewarmed physiological solution maintained in a bain-marie at the appropriate temperatures for treatment. At the end of each treatment, larvae were quickly transferred into 60 ml of physiological solution precooled to 10°C. The microscopic assessment of larval inactivation was performed after 1 h at room temperature in the physiological solution.

After each treatment, larval lengths in millimeters were measured. Data from each trial, group, and time interval were statistically analyzed using *T* tests to cluster larvae by length and to assess the inactivation rate (IR = time necessary for the inactivation of 1% of larvae under fixed treatment conditions) for each length group. IR values were expressed in hours for marinating trials and in minutes for thermal treatments. The discriminant mean lengths for marinating treatments were 10, 14, and 16 mm, whereas the discriminant mean lengths for thermal treatments were 15, 18, 20, and 22 mm.

26.3 Results

All examined larvae belonged to the *Anisakis* genus. The relationships between IR values and larval lengths for the marinating trials are reported in Fig. 26.1. Larval resistance to the harsher treatment (group A) was directly proportional to length, while shorter larvae were more resistant to the combined effect of NaCl and organic acids in the milder treatments (group B and C).

With regard to thermal treatments, Fig. 26.2 indicates, as mentioned above, that 47°C produced an amount of larval inactivation within an appropriate time interval for appreciating the variability of responses. The IR values as a function of length and the IR mean values for all larvae (IR tot) are reported in Fig. 26.3. Only the group with a mean length of 22 mm had an IR value greater than IR tot.

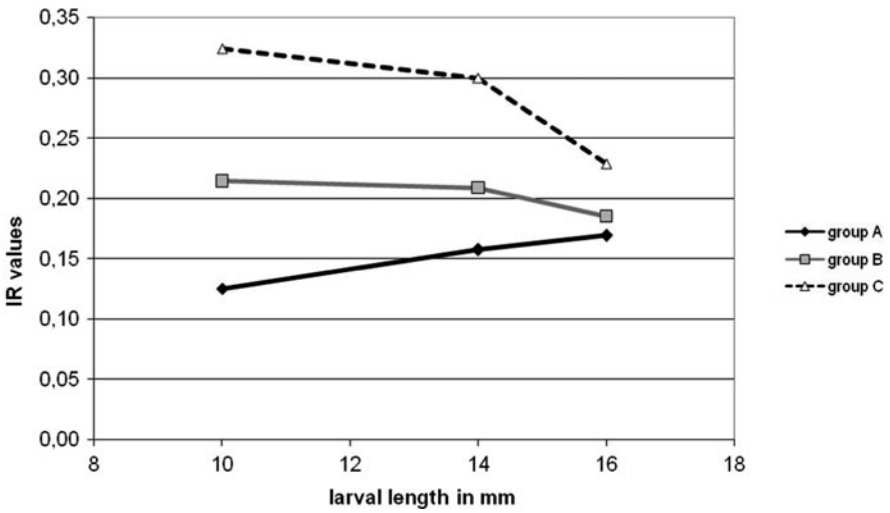

Fig. 26.1 IR values of each group subjected to different marinating treatments (groups A, B, and C) with respect to larval length

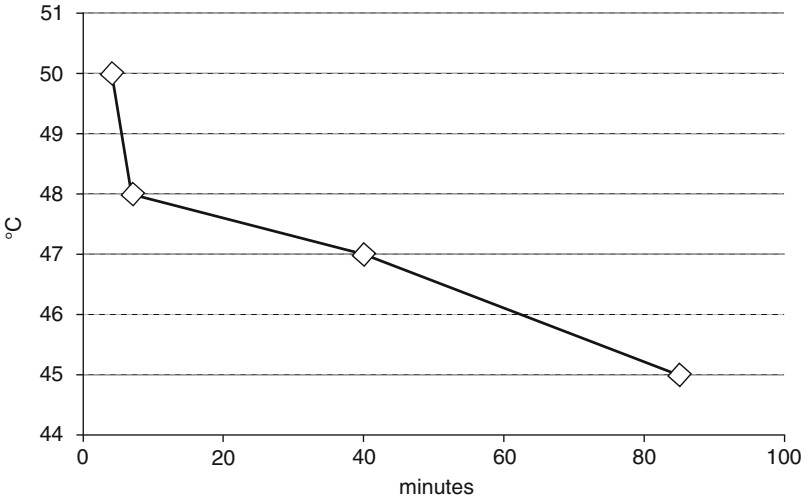

Fig. 26.2 Temperature:time ratios resulting in inactivation of 100% of the larvae

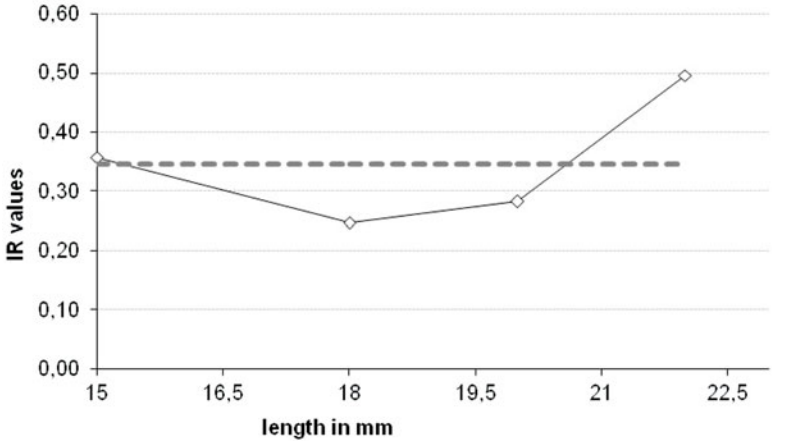

Fig. 26.3 Relationship between IR and larval length at 47°C. The *dotted gray line* indicates the IR mean value for all considered larvae (IR tot)

26.4 Discussion

This study highlights the greater resistance that longer *Anisakis* larvae have to a marinade with high concentrations of NaCl (13%), acetic acid (5%), and citric acid (5%), whereas shorter larvae were more resistant to mild marinating treatments. In the case of thermal treatments, the group with a mean length of 22 mm had the highest resistance. However, the correlation between length and IR values was not

linear since the 15-mm group had a greater IR value than did the 18- and 20-mm groups (Fig. 26.3).

These data demonstrate that a precise relationship between larval length and resistance to technological stressors cannot be defined, suggesting there is individual variability. The wide variability of responses to marinating and thermal treatments, as well as the great heterogeneity of larval lengths within the same fish species harvested from the same zone, requires the review of data on the inactivation of anisakid larvae. In this regard, the individuation of equivalent treatment to freezing at −20°C should be performed with particular caution.

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Chapter 27
Prevalence of Verocytotoxin-Producing *E. coli* in Sheep Meat at a Slaughterhouse

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Abstract Sheep play important roles in the spread of pathogenic *Escherichia coli* that can cause severe diseases in humans. The aim of the present study was to evaluate the prevalence of verocytotoxin-producing *E. coli* (VTEC) in various samples from Sarda sheep using a molecular screening test and to define the virulence profiles of isolates using multiplex PCR. A total of 380 different ovine samples (fleece, carcass surface, and mucosal gut) were analyzed by direct PCR screening for the presence of stx_1 e stx_2 genes. Virulence factors (stx_1, stx_2, and *eae*) from the strains, isolated by immunomagnetic separation (IMS)-based cultivation techniques (e.g., CT-SMAC, CT-RMAC, and EHLY agar), were determined by multiplex PCR. An overall prevalence of 11.1% (adults 14%, lambs 7.8%) was found by direct PCR. VTEC occurrence was 18.9% in fleece, 14.7% on carcasses, and 10.5% in mucosal gut. According to the multiplex-PCR results, the following values were obtained: 43.4% VTEC (stx_1, stx_2, $stx_1 + stx_2$, $stx_2 + eae$, and $stx_1 + stx_2 + eae$), 30.3% EPEC (stx_1–/stx_2–/*eae*+), and 26.4% negative for all genes examined (stx_1–, stx_2–, *eae*–).

Keywords *E. coli* • PCR • Sheep • VTEC

27.1 Introduction

Verocytotoxin (VT)-producing *Escherichia coli* (VTEC) are zoonotic agents that cause a potentially fatal human illness; its clinical spectrum includes diarrhea, hemorrhagic colitis (HC), and hemolytic–uremic syndromes (HUS) (Karmali et al. 2010). The presence of VTEC in foods is a serious public health concern

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as demonstrated by outbreaks that occurred in many industrialized countries (Bonardi and Leccese 2006). Cattle are the main reservoir of VTEC, but small ruminants also play an important role in the transmission of these pathogens to humans. Small ruminants represent one of the more important reservoirs of VTEC. An increased prevalence of a wide variety of serotypes in the gut of clinically healthy sheep has been described (Zweifel et al. 2004; Urdahl et al. 2001; Blanco et al. 1996).

Epidemiological studies have demonstrated that some strains of VTEC isolated from healthy sheep have genetically distinct subtypes of Shigatoxine (stx_1c and stx_2d) (Pierard et al. 1998). Shigatoxine subtypes are not commonly recovered from the feces of patients experiencing HC or HUS, but are isolated from cases of uncomplicated diarrhea. Furthermore, lambs, as compared to adult sheep, carry a greater variety of atypical-VTEC serotypes that temporarily colonize intestinal tracts. These differences may be related to the combined effect of age, physiological differences in intestinal tracts, diets, and/or different immune response to these pathogens (Djordjevic et al. 2004). The aim of this study was to determine the prevalence of VTEC in sheep slaughtered in Sardinia, where approximately three million heads are reared, to further molecularly characterize the pathogenic profiles of the isolated strains.

27.2 Materials and Methods

27.2.1 Sample Collection

From three slaughterhouses located in Sardinia, 380 samples from 95 animals (50 sheep and 45 lambs) were collected that included the following matrices: (a) scrapes of the colon and the rectum mucosa (10 g, after evisceration); (b) skin from the abdomen and the chest (20 cm^2 next to the incision line); and (c) carcass surfaces by sponge-swab (300 cm^2) of regions of the chest, abdomen, and leg.

27.2.2 Rapid Screening and Strain Isolation

A 10-g portion of each sample was diluted tenfold in 90 ml of modified Tryptone Soya Broth (m-TSB) supplemented with 20 mg/l novobiocin. Enriched broths were incubated at 37.5°C for 18–20 h. Rapid molecular screening was used to evaluate the presence of *vtx* genes using the PCR method of the EU Food Project with modifications. One milliliter of each sample was subjected to (1) DNA extraction using the resin Chelex-100 (Bio-Rad, USA) and (2) amplification of stx_1 and stx_2 with the primer pair MK1 (5′-TTTACGATAGACTTCTCGAC-3′) and MK2 (5′-CACATATAAATTATTTCGCTC-3′) that resulted in a PCR product of

230 bp. Positive enrichments from the PCR screening were submitted to manual IMS performed using Dynabeads anti-*E. coli* O157, O26, O103, O111, and O145 (DY 71004; Dynal, Oslo, Norway) as per the manufacturer's instructions. After the IMS, a 50-μl suspension was streaked onto the following media: CT-SMAC (cefixime tellurite sorbitol MacConkey agar) for the detection of the O157 serotype, CT-RMAC (cefixime tellurite rhamnose MacConkey agar) for the O26 serotype, and EHLY agar for the O103, O111, and O145 serotypes, and incubated at 37°C for 24 h. Colonies with typical characteristics were subjected to phenotypic identification using the API 20 E (20100; bioMérieux) system. Furthermore, the cultures of the O157 strain were tested using an *E. coli* O157 Latex test kit (DR 620; Oxoid).

27.2.3 Molecular Characterization of the Isolates

Fifty-three strains from the different matrices were subjected to multiplex PCR for the detection of stx_1, stx_2, and *eae* (Paton and Paton 1998; EFSA Report 2010) using the primers stx_1F (5′-ATAAATCGCCATTCGTTGACTAC-3′), stx_1R (5′-AGAACGCCCACTGAGATCATC-3′), stx_2F (5′-GGCACTGTCTGAAACTGCTCC-3′), stx_2R (5′-CGCCAGTTATCTGACATTCTG-3′), *eae*AF (5′-GACCCGGCACAAGCATAAGC-3′), and *eae*AR (5′-CACCTGCAGCAACAAGAGG-3′), which resulted in the amplification of 180-, 285-, and 384-bp products, respectively.

27.3 Results

According to the preliminary screening test, the overall prevalence of VTEC was 11.1% (14% in sheep and 7.8% in lambs). In particular, 18.9% of the skin, 14.7% of the carcass, and 10.5% of the mucosa samples were positive (Table 27.1). The strains subjected to molecular characterization were (1) CT-SMAC, n.24, isolated from different matrices in different plants, (2) CT-RMAC, n.18, isolated from skin samples in a single plant, and (3) EHLY agar, n.11, from different matrices in different plants. All strains were confirmed by phenotypic identification as *E. coli*, but none of the isolates were positive for the O157 serotype by the *in vitro* agglutination test. Based on the multiplex PCR results, the isolates (Fig. 27.1) belonged to three groups (Tables 27.2a and 27.2b): (1) VTEC, 43.4% (n.23), with

Table 27.1 VTEC prevalence (%) as determined by a PCR screening method in relation to the matrix and animal age

	Matrix			
	Skin	Carcass	Mucosa	Feces
Sheep	13.7	9.5	6.3	–
Lamb	5.3	5.2	4.2	–
Total	18.9	14.7	10.5	–

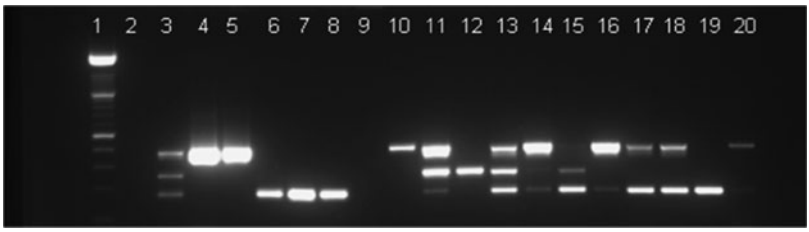

Fig. 27.1 Multiplex-PCR products (*stx*$_1$, *stx*$_2$, *eae*). *Lane 1*: marker (100-bp ladder), *lane 2*: negative control, *lane 3*: positive control (180, 285, and 384 bp), *lanes 4–20*: ovine isolates

Table 27.2a Prevalence (%) of VTEC-positive strains as determined by multiplex PCR (*stx*$_1$, *stx*$_2$, *eae*)

	stx$_1$			*stx*$_2$			*eae*		
	Skin	Mucosa	Carcass	Skin	Mucosa	Carcass	Skin	Mucosa	Carcass
Sheep	–	1.9 (1[a])	–	–	–	3.8 (2)	1.9 (1)	1.9 (1)	11.3 (6)
Lamb	–	–	–	3.8 (2)	–	–	3.8 (2)	3.8 (2)	7.5 (4)
Total	–	1.9 (1)	–	3.8 (2)	–	3.8 (2)	5.7 (3)	5.7 (3)	18.9 (10)

[a]The number in brackets indicates the relationship between the matrix and age

Table 27.2b Prevalence (%) of VTEC-positive strains as determined by multiplex PCR (*stx*$_1$, *stx*$_2$, *eae*)

	stx$_1$/*stx*$_2$			*stx*$_2$/*eae*			*stx*$_1$/ *stx*$_2$/ *eae*		
	Skin	Mucosa	Carcass	Skin	Mucosa	Carcass	Skin	Mucosa	Carcass
Sheep	1.9 (1[a])	7.5 (4)	–	–	1.9 (1)	5.7 (3)	–	1.9 (1)	–
Lamb	–	–	5.7 (3)	–	–	–	7.5 (4)	1.9 (1)	–
Total	1.9 (1)	7.5 (4)	5.7 (3)	–	1.9 (1)	5.7 (3)	7.5 (4)	3.8 (2)	–

[a]The number in brackets indicates the relationship between the matrix and age

a profile of *stx*$_1$ +, *stx*$_2$ +, *stx*$_1$ +/*stx*$_2$ +, *stx*$_2$ +/*eae* +, *stx*$_1$ +/*stx*$_2$ +/*eae* +, (2) EPEC, 30.3% (n.16), with the intimin gene only (*stx*$_1$–/*stx*$_2$–/*eae* +), and (3) Non-producer, 26.4% (n.14), negative for all of the genes (*stx*$_1$–, *stx*$_2$–, *eae*–). No correlation was found between the pathogenic profiles and the matrix or category of the animals from which the strains were isolated.

27.4 Discussion

The results from this preliminary molecular screening showed an overall occurrence of VTEC (11.1%) that was higher than that previously reported for O157:H7 (0.3–7%) (EFSA Report 2010). In particular, skin was confirmed as the major source of contamination (Ogden et al. 2005) because of the probable fecal origin of VTEC. In fact, surface contamination of carcasses frequently correlated with

visible fecal contamination, especially in lambs that may have soft stools and sometimes diarrhea. Moreover, a lack of GHP application at slaughterhouses can occur during intensive production. The nondetection of VTEC in feces is likely due to limited sensitivity of the method, considering the presence of a polymicrobial flora and other PCR-inhibiting factors, such as bile salts.

The molecular method used here was useful as a rapid screening test for direct detection of VTEC in food matrices. This method showed a greater sensitivity than culture-based methods, but the latter are essential for confirming the presence of the pathogen and for defining their pathogenic profiles. Furthermore, the application of multiplex PCR allowed for the detection of different pathotypes of considerable public health interest. These results, although preliminary, confirm the complexity of ovine *E. coli* pathogenic profiles. Further molecular investigations will be performed to both determine serotypes and detect other pathogenic factors (Paton et al. 2004).

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Chapter 28
Ultrasonographic Assessment of Thawing in Sea Breams (*Sparus aurata*)

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Abstract To assess sea bream (*Sparus aurata*) thawing, two ultrasonographic (US) protocols were used. The first protocol was used on 15 subjects at three different times (1) immediately after capture, (2) after the first thaw, and (3) after the second thaw. A general-purpose device equipped with a linear high-frequency probe was used. On the B-mode-Real Time images, quantitative analysis of the epiaxial muscle gray levels was made. For the second protocol, a quantitative US (QUS) device was used on three groups of fish samples (fresh, thawed once, and thawed twice), each composed of five subjects. Speed of sound, bone ultrasound attenuation, bone mineral density, and quantitative ultrasound index were measured. Data were statistically compared using ANOVA ($P < 0.05$). Gray levels differed significantly among the three control times. QUS data differed significantly between the fresh and thawed groups. US and QUS appear to be promising techniques for assessing fish thawing.

Keywords Ultrasonography • Sea bream • Thawing

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Abbreviations

BMD Bone mineral density
BUA Bone ultrasound attenuation
QUI Quantitative ultrasound index
SOS Speed of sound

28.1 Introduction

Assessment of fish thawing is usually made through direct inspection by experienced technicians; however, in some cases, the assessment may be difficult or impossible, particularly in prepared and transformed products (fillets, tails, slices, etc.). Imaging diagnostic techniques may provide noninvasive morphological and structural information on analyzed tissues. Currently, diagnostic imaging has been used to examine certain characteristics of parmigiano reggiano cheese and raw ham (prosciutto crudo di Parma) (Orlandini et al. 1985) to evaluate food adulteration, identify fish species (Anastasio et al. 2002; Anastasio et al. 2004; Cortesi 2005), and verify food freshness (Meomartino et al. 2006). In fish products, ultrasonography (US) was demonstrated to be a rapid method to assess (a) changes related to storage conditions, (b) meat and fat tissue distribution, and (c) anatomical marks useful to prove fraud (Anastasio et al. 2004; Cortesi 2005; Meomartino et al. 2006). It is well known that, following freezing and thawing, fish soft tissues undergo ultrastructural changes characterized by sarcolemmal damage and clear interfibrillar spaces filled by intracellular fluids (Ayala et al. 2005). As a consequence, soft tissues become more "rigid," which improves impedance and can be determined via increased reflection and speed of the ultrasounds during their progression through the tissues. The purpose of the present study was to assess whether US is a valuable method to assess changes in thawed sea bream (*Sparus aurata*) tissues.

28.2 Materials and Methods

The study was organized in two steps. In the first step, the epiaxial muscles of 15 sea breams, weighing 400–500 g and coming from an offshore fish farm in the Pozzuoli Gulf (Naples), were examined using US. To make the US protocol simpler and more reproducible, three transverse scans were made on each subject: the first at the middle of the lateral line and the others at the middle of the cranial and caudal halves of the lateral line. The US study was performed on five fresh (8 h after capture) sea breams, five subjects after a first thawing, and five subjects after

a second thawing. Thawing was performed in both cases at 4°C for 18 h. A general-purpose device (Mindray 6600) equipped with a high-frequency (10 MHz) linear probe was used for US studies. During all studies, US settings were always the same. On all the images, means and median gray levels were measured at the central myomer using a commercial graphic editing program (Adobe Photoshop 7).

In the second step, 15 sea breams, of the same weight and origin, were used: five fresh, five after a first thawing, and five after a second thawing (both thawings at 4°C for 18 h). A quantitative US (QUS) device (Hologic Sahara) equipped with two low-frequency (0.6 MHz) probes and currently used for Calcaneal US densitometry in humans was used. During the studies, speed of sound (SOS), bone ultrasound attenuation (BUA), bone mineral density (BMD), and quantitative ultrasound index (QUI), a value obtained by the ratio of the two above-mentioned values, were measured. To obtain the measurement at the level of the lateral line of the fish, the body of the sea bream was cut into two pieces, a cranial piece and a caudal piece. The data were statistically analyzed using the ANOVA test (Tables 28.1 and 28.2). Significance level was set at P less than 0.05.

Table 28.1 Mean (± standard deviation [SD]) and median values (± SD) of gray levels at the central myomer and P values of the ANOVA comparison among the three US studies

	1 Fresh	2 After the first thawing	3 After the second thawing
Mean (± SD)	12.79 (±5.06)	19.56 (±5.07)	27.23 (±3.64)
Median (± SD)	9.29 (±6.01)	18.17 (±5.76)	23.26 (±3.59)
*P**	1 vs. 2	2 vs. 3	1 vs. 3
Mean	<0.0001	<0.0001	<0.0001
Median	<0.0001	<0.0001	<0.0001

*Significance level $P < 0.05$

Table 28.2 Mean (± SD) of SOS, BUA, BMD, and QUI values and P values of the ANOVA comparison among the three groups of sea breams

	1 Fresh	2 After the first thawing	3 After the second thawing
SOS (± SD)	1,552.11 (±6.2)	1,556.68 (±16.3)	1,550.83 (±9.7)
BUA (± SD)	13.31 (±22.1)	57.48 (±37.9)	76.81 (±16.8)
BMD (± SD)	0.37 (±0.05)	0.50 (±0.11)	0.53 (±0.05)
QUI (± SD)	70.68 (±8.41)	91.86 (±15.9)	96.47 (±8.12)
P*	1 vs. 2	2 vs. 3	1 vs. 3
SOS	<0.428	<0.253	<0.934
BUA	<0.0001	<0.07	<0.0001
BMD	<0.0001	<0.435	<0.0001
QUI	<0.0001	<0.413	<0.0001

*Significance level $P < 0.05$

SOS speed of sound, *BUA* bone ultrasound attenuation, *BMD* bone mineral density, *QUI* quantitative ultrasound index

28.3 Results

Echogenicity of the central myomer increased in thawed subjects, and the mean and median values of the gray levels were significantly different in the three US studies of the first step (Table 28.1). In the second step, QUS BUA, BMD, and QUI values were significantly higher in thawed sea breams. No significant differences were found between fish thawed once and fished thawed twice. SOS showed no statistical differences among the three groups (Table 28.2).

28.4 Discussion

In a previous study, gray-level analysis of the central epiaxial myomer demonstrated to be a simple and useful method to assess fish freshness (Meomartino et al. 2006). In the present study, the method was simplified, and the modifications were more reproducible. A marked increase in echogenicity with a more significant difference among the fresh and thawed sea breams was evident. No common values were observed between fresh and twice-thawed subjects, but there were many overlapping values between fresh and once-thawed fishes.

To verify whether the differences between fresh and thawed tissues demonstrated by US images could be expressed numerically, comparisons among fresh, once-thawed, and twice-thawed sea breams were made using QUS. This method, with the exception of the SOS values, demonstrated a significant difference between fresh and thawed fishes but not between once- and twice-thawed sea breams. This lesser sensitivity of the QUS, compared to the US imaging, could be explained by at least two reasons (a) the tissues studied were soft tissues in which US progresses virtually at the same speed and (b) the device used in this study had been designed for human bone density assessments, and its probe used a very low frequency that was likely unable to detect the small differences in the SOS and impedances present in the examined tissues.

The study demonstrated a direct correlation between echogenicity and hydration status of fresh sea breams. Since thawing causes both sarcolemmal damage and intracellular fluid loss, tissue stiffness is produced, which affects tissue density and ultrasound speed. These changes cause an increase in tissue echogenicity, which may be assessed subjectively but could also be translatable numerically. The preliminary findings are promising and show that US studies could be valuable tools to recognize thawed fish. US could be used for quality and safety controls performed for official and private purposes. Dedicated devices and cutoff values for several fish species and products are needed before US studies may be proposed and routinely used.

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Chapter 29
Quantification of Patulin in Piedmont Cheese by HPLC: Preliminary Note

D. Pattono, M. Pazzi, G. Zeppa, and T. Civera

Abstract The aim of the present study was to assess patulin content in traditional cheeses, purchased from the retail trade. Samples for analysis were taken from both the inner part of the cheese and the rind. Only one out of 25 inner cheese samples contained patulin, while eight out of 25 rind samples were positive. The level of patulin in the inner part of the cheese was 26.6 ppb. In the rind, the contamination level ranged between 15.4 ppb and 460.8 ppb. Even though the rind is not considered edible, many consumers eat it. In addition, one cannot dismiss the possible migration of toxinogenic molds from the rind to the inner part of the cheese caused by blowing or other defects. For this reason, careful control of all ripening conditions is recommended.

Keywords Cheese • HPLC • Patulin • Traditional products

Abbreviations

HPLC	High-pressure liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
RDS	Relative standard deviation

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29.1 Introduction

In recent years, mycotoxins have gained increasing importance in public health. Nowadays, in addition to aflatoxins and ochratoxins, other mycotoxins such as patulin are considered of relevant interest. This mycotoxin is produced by several fungal species: *Aspergillus* spp., *A. clavatus*, *Penicillium expansum*, *P. clavigerum*, *P. griseofulvum*, *Byssoclamis* spp., *B. nivea*, and *B. fulva*. It is considered neurotoxic, hembriotoxic, and teratogenic (Dombrink-Kurtzman and Balckburn 2005). It has been classified by the International Agency for the Research on Cancer as category 3, even though its carcinogenic activity on animal models has been demonstrated (Cunha et al. 2009). The law limits of patulin in foodstuffs have been established only for fruits such as apples and apple-based products (Commission Regulation 1881/2006). However, patulin has been detected in many other food items: bread and baked products and meat products (López-Díaz et al. 1996; Erdogan et al. 2003). Furthermore, toxin-producing strains can be used as natural starter cultures or can be present in cheese manufacturing, seasoning, and ripening environments; however, few data are available for dairy products (López-Díaz et al. 1996; Erdogan et al. 2003; Kokkonen et al. 2005).

The aim of this work is the identification and quantification of patulin in a traditional, hand-made, semihard cheese named "Toma Piemontese." Such a type of cheese has been considered because it is generally manufactured and stored with procedures and in environments not always carefully controlled. All samples were acquired from the retail trade; some of them showed defects and mold growth on the surface.

29.2 Materials and Methods

The study has considered 25 semihard cheeses (inner parts and rind) produced with raw cow milk with a cheese-making technique usually applied for "Toma Piemontese." The milk is coagulated by the addition of bovine liquid rennet at temperatures between 32 and 35°C. The curd is cut twice to produce granules about the size of maize grains. After draining, the curd is put in molds, pressed for approximately 36 h, and then dry salted or salted in brine.

The identification and quantification of patulin were performed using an HPLC apparatus.

Patulin standards were purchased from Fluka (Buchs, Switzerland). All solvents were of HPLC grade (Merck, Darmstad, Germany). The water was filtered by a MilliQ System (Millipore, Billerica, MA, USA). For extraction and quantification, the protocol of Kokkonen et al. (2005) was used. The confirmation of the results

was made by gas chromatography (GC) following the protocol of Cunha et al. (2009). The LOD and LOQ were calculated as stated by Miller and Miller (2000).

29.3 Results

Two analyses, each with five replicates, were carried out to assess recoveries. At 100 ppb, the percentage of recovery was 87.1 ± 6.6 (RSD 7.7%) and was 89.2 ± 5.2 (RDS 5.9%) at 500 ppb. The calibration curve on six points was linear in the range considered (from 10 ppb to 500 ppb) with a relative regression coefficient (R^2) of 0.9989. LOD was 2.1 ppb and LOQ 7.7 was ppb. The results of the analysis are reported in Table 29.1.

Only one sample of the inner part (4% of examined samples) was positive with a content of 26.6 ppb. A different situation was highlighted for the rind. Eight out of 25 samples were positive (32% of examined samples). The amounts were between 15.4 and 460.8 ppb. Positive samples were confirmed by GC analysis.

Table 29.1 Patulin content in cheese (rind)

Sample number	Quantity of patulin in rind (ppb)
1	n. d.
2	43.8
3	460.8
4	235.3
5	77.3
6	n. d.
7	n. d.
8	68.0
9	184.6
10	15.4
11	n. d.
12	n. d.
13	n. d.
14	n. d.
15	n. d.
16	n. d.
17	n. d.
18	n. d.
19	n. d.
20	n. d.
21	n. d.
22	n. d.
23	n. d.
24	57.9
25	n. d.

No patulin was detected in the inner part of the cheese except for sample 8 (26.6 ppb; see Results section). *n.d.* not detected

29.4 Discussion

As reported, patulin is a mycotoxin produced by several fungal species and was detected in many food items; however, unlike what other authors have found (Erdogan et al. 2003; Kokkonen et al. 2005; Taniwaki et al. 2009), we detected patulin in cheese samples. In particular, we detected patulin in samples taken from the rind. However, when these results are compared with the amounts established as maximum limits by the Reg. 1881/2006 (i.e., from 10 to 50 μg/kg), it is clear that little concern exists for consumer health because the mycotoxin has been detected mainly in the rind.

Conversely, the rind is often eaten by consumers even when covered with mold, as they consider such a product more "typical and wholesome" (Sulyok et al. 2010). Therefore, the daily intake of patulin can reach higher levels, as it is also present in other foods (e.g., fresh and dried fruits, bread, and cereals).

Another aspect to be taken into account is the possibility that the fungal colonies can pass from the rind into the inner part of the cheese, mainly when defects, such as early blowings, late blowings, etc., are present. It can also be supposed that the mycotoxin itself can spread in the cheese as stated in recent studies (Sulyok et al. 2010).

Recent studies have demonstrated the presence of patulin in samples without clearly visible mold (Rychlik and Schieberle 2001; Sulyok et al. 2007, 2010).

Certainly, the food manufacturing and ripening environment is the main source of contamination of cheese. In this respect, it has been demonstrated that humidity, temperature, and oxygen tension can play important roles (Erdogan et al. 2003; Taniwaki et al. 2009). Wood tables as well, where the cheeses are laid during the ripening period, could be involved in the contamination, as has been observed in meat products (López-Díaz et al. 1996; Søresen et al. 2008)

In conclusion, a strict surveillance of all the steps in cheese making and ripening is essential to ward off the risk of mycotoxin contamination.

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Part V
Husbandry and Zootechnic

Chapter 30
Association Analysis Between DNA Markers and Number of Functional Teats in Italian Large White Pigs

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Abstract The aim of this work was to study the association between five single-nucleotide polymorphisms (SNPs), each in one of five genes (*CTSL*, *EPOR*, *ESR2*, *GPX5*, and *STAT5B*), and functional teat number (FTNUM). Each marker was genotyped in at least 940 Italian Large White sows. All SNPs segregated in the analyzed breed with minor allele frequencies ranging from 0.18 (*CTSL* and *EPOR*) to 0.40 (*ESR2*). The genotyped sows were categorized into two classes based on their FTNUM: FTNUM equal to 14 (59% of the samples) and FTNUM greater than 14 (41%). Differences in allele frequencies between the two classes were statistically significant ($P < 0.01$) for the *GPX5* polymorphism.

Keywords Polymorphisms • Functional teat number • Sows • Italian Large White

Abbreviations

ANAS	National Association of Pig Breeders
FTNUM	Functional teat number
ITLW	Italian Large White pig breed
MAF	Minor allele frequency
MAS	Marker assisted selection
QTL	Quantitative trait loci
SNP	Single nucleotide polymorphism
UTR	Untranslated region

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30.1 Introduction

The number of functional teats (FTNUM) in sows affects piglet survival and reproductive efficiency that, in technical terms, is measured by the number of piglets weaned per sow per year. The number of teats is a discontinuous, multifactorial trait with medium–low heritability (Nicholas 1998). The Italian Large White pig breed (ITLW) was selected for FTNUM using thresholds, and only pigs with at least 14 functional and accessible teats (regularly spaced, well protruded, and with normal and functional nipples) are registered in the breed Herd Book (maintained by the National Association of Pig Breeders, ANAS; http://www.anas.it). Marker-assisted selection (MAS) could be an important tool for genetic improvement of FTNUM (Buske et al. 2006). At present, 74 quantitative trait loci (QTL) for the number of teats have been reported, indicating a polygenic influence on this trait (PigQTL Database; http://www.genome.iastate.edu/cgi-bin/QTLdb/SS/summary; release of April 14, 2010). Considering that litter size is the most important reproductive trait in sows, it might be interesting to analyze whether alleles in candidate genes can be used as markers to improve FTNUM. The aim of this study was to evaluate association between polymorphisms within five genes and FTNUM in ITLW.

30.2 Materials and Methods

Hair samples were collected from 1,112 ITLW sows from five farms located in the Emilia-Romagna and Lombardy regions. The samples were used for genomic DNA extraction and genotyping of five single-nucleotide polymorphisms (SNPs) within five different genes. Table 30.1 reports information about the targeted genes and the analyzed SNPs. The genotyping was performed using the Sequenom Iplex™ GOLD Platform system. A total of 5,215 genotypes were generated (Table 30.2). To check the quality of the results, approximately 2% of the samples were analyzed in duplicate, and Hardy–Weinberg equilibrium was verified by chi-square test. FTNUM was determined through visual inspection. This information was provided by ANAS that records this trait for all ITLW animals registered in the Herd Book. The sows were categorized into two groups based on their FTNUM (1) sows with 14 teats (minimum requested by the Herd book) and (2) sows with more than 14 teats. For each polymorphism, differences in allele and genotype frequencies between the two groups of sows were tested using the FREQ procedure of SAS (version 9.2). To reduce the possibility of type I errors (false positives), nominal P values were corrected for multiple testing using the Bonferroni correction.

30.3 Results

An association study between five polymorphisms located in different genes (Table 30.1) and FTNUM was carried out. All polymorphisms segregated in ITLW. Minor allele frequency (MAF, Table 30.2) ranged from 0.18 (allele AJ315771:g.5325 T of *CTSL* and allele EU4077778:g.2373 T of *EPOR* genes) to 0.40 (allele AF164957:c.949 G of the *ESR2* gene). As genetic markers were informative (Table 30.2), it was possible to perform an association study with FTNUM. Considering the entire analyzed population, the mean and standard deviation of these traits were equal to 14.57 ± 0.76. In particular, 59% of sows had 14 FTNUM (group 1), and the remaining 41% had 15–17 FTNUM (group 2). The sows of the two groups had similar inbreeding mean values and years of birth (between 2002 and 2007). The classification of the sows into two groups made it possible to apply a statistical analysis to the categorical variables. Table 30.2 reports MAF in the entire sample and in the two groups of sows for the five analyzed polymorphisms. All SNPs were in Hardy–Weinberg equilibrium in both groups of sows ($P > 0.05$). The *CTSL*, *EPOR*, *ESR2*, and *STAT5B* marker genes were not associated with FTNUM. A statistically significant difference of allele

Table 30.1 Details of the analyzed polymorphisms and references

Gene name, symbol	Accession number: polymorphism	Chromosome, gene region, amino acid change	References
Cathepsin L, *CTSL*	AJ315771: g.5325 C > T	10, exon 5, silent	Fontanesi et al. (2010)
Erythropoietin receptor, *EPOR*	EU4077778: g.2373 C > T	2, intron 4	Vallet et al. (2005)
Estrogen receptor 2, *ESR2*	AF164957: c.949 G > A	1, exon 5, p.V317M	Muñoz et al. (2004)
Glutathione peroxidase 5, *GPX5*	AF124818: g.1939 C > T	7, 3′-UTR	Bertani et al. (1999)
Signal transducer and activator of transcription 5B, *STAT5B*	DQ144238: g.191A > G	12, intron 14	Ballester et al. (2006)

Table 30.2 Minor allele frequency (MAF) of the polymorphisms analyzed in the entire sample and in the two groups of sows based on their teat number

Genes	No. of analyzed sows	MAF – entire data set	MAF – Group 1 FTNUM = 14	MAF – Group 2 FTNUM >14	Allele frequency difference 1 vs. 2, P[a]
CTSL	1,086	0.18 (T)	0.18	0.19	0.7112
EPOR	1,027	0.18 (T)	0.17	0.19	0.1236
ESR2	1,094	0.40 (G)	0.40	0.41	0.5686
GPX5	940	0.36 (T)	0.34	0.40	0.0087
STAT5B	1,091	0.20 (G)	0.20	0.21	0.5528

[a]Probability of the comparison (P)

frequencies (P nominal value = 0.0087; P = 0.0435 after Bonferroni correction) was obtained for the *GPX5* SNP.

30.4 Discussion

We analyzed polymorphisms in five candidate genes for prolificacy of sows to detect association with FTNUM of ITLW. *CTSL* encodes a lysosomal proteinase with a potential role in embryo implantation, while *EPOR* controls the differentiation of red blood cells in the fetus and is a candidate for uterine capacity (Vallet et al. 2005). *ESR2* is essential for ovulation and embryo development (Muñoz et al. 2004). *STAT5B* encodes a protein involved in signal transduction of hormones and cytokines (Ballester et al. 2006). *GPX5* encodes a glutathione peroxidase involved mainly in sperm quality and male fertility, and it was a considered a positional candidate gene for fertility due its location in a chromosomal region encompassing QTL for reproductive traits. The analyzed polymorphisms, except for the *CTSL* (Fontanesi et al. 2010) and *ESR2* SNPs (Dall'Olio et al. 2010), have not been investigated in ITLW before. All polymorphisms segregate in this breed; for this reason, it was possible to use these markers in association studies. The results indicated that the *GPX5* SNP is associated with FTNUM variability in ITLW ($P < 0.01$). The allele that showed a favorable effect on teat numbers (*GPX5:* AF124818:g.1939 T) was the less frequent in the breed (group 1 = 0.34, group 2 = 0.40, Table 30.2). From a physiological point of view, it seems that there is no direct relation between GPX5 function and the number of teats. The analyzed SNP of the *GPX5* gene (Bertani et al. 1999), located in the 3′-UTR, could affect mRNA stability even if this role seems unlikely. Therefore, considering also the function of this gene, it is possible that *GPX5* does not have a direct effect on the number of teats, but its association may be due to linkage disequilibrium with a functional allele within another gene on chromosome 7. However, the obtained result opens up interesting perspectives, as this trait is related to the mothering ability of the sows. We are evaluating whether the same markers analyzed in this study are associated with prolificacy in Italian Large White sows.

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Chapter 31
White Sturgeon (*Acipenser transmontanus*) Optimum Feeding Rates at Weeks 6 and 10 After Initiation of Feeding

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Abstract The effects of feeding rates on growth performance and body proximate composition of white sturgeon juveniles 6 and 10 weeks after initiation of feeding were assessed. Two 1-week-long trials with six different feeding rates, replicated four times, were carried out. The feeding rates were 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0% body weight (bw)/day (d) in the first trial and 0.8, 1.6, 2.4, 3.2, 4.0, and 4.8% bw/d in the second trial. Sixty fishes with 2.27 $\pm$ 0.9 g (mean $\pm$ SD) initial body weight were used in the first trial, and 25 fishes with 15.78 $\pm$ 4.4 g initial body weight were used in the second trial. The juveniles were kept at 18–19°C in small circular tanks (66 cm in diameter and 27 cm in water height) containing 90 L water and 3 L/min flow and were fed a commercial salmonid soft-moist feed using automatic feeders. Mortality was almost zero (only one fish died in the first trial, which was unrelated to feeding rates). Specific growth rate (SGR), feeding efficiency, and body composition were significantly ($P < 0.05$) affected by feeding rates. Broken-line analysis of SGR indicated that the optimum feeding rates of white sturgeon juveniles were 6.5 $\pm$ 0.4% and 3.8 $\pm$ 0.1% bw/d for the fifth and the 10th week after initiation of feeding.

Keywords Aquaculture • Feeding rate • White sturgeon

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31.1 Introduction

White sturgeon (*Acipenser transmontanus*) is one of the most suitable *Acipenser* species for aquaculture production (Hochleithner and Gessner 2001). White sturgeon is reared for caviar production and for row and smoked fillets, whose market has been continuously increasing over the years (Bronzi et al. 1999). Feeding rate is one of the most important factors influencing growth and feed efficiency of fishes (Brett 1979). Knowledge of the optimum feeding rate of a fish species is important for the success of its aquaculture production, because it allows the farmers to obtain the largest growth per unit feed cost, maintain good water quality, minimize environmental stress, and, thus, maximize production efficiency. Several studies have been conducted to determine the optimum feeding rates of white sturgeon (Hung and Lutes 1987; Hung et al. 1989, 1995, Deng et al. 2003). There is, however, no information on the optimum feeding rates of white sturgeon larvae (2.2–15.8 g) from 6 to 10 weeks after initiation of feeding (Hung and Deng 2002).

The objectives of the present study were to determine the optimum feeding rates and to investigate the effects of feeding rates on the growth performances and proximate body compositions of white sturgeon larvae at the 6th and 10th weeks after initiation of feeding. This period of a sturgeon's life is particularly critical because fishes overfed or underfed may be more susceptible to pathologies that could contribute to the high mortality rates typical at this age.

31.2 Materials and Methods

One-day posthatch yolk-sac white sturgeon larvae (10,000) from a single progeny were kept in two large, circular fiberglass tanks (150 cm diameter by 45 cm height with 675 L water) that were supplied with 10–18 L/min per tank of degassed groundwater with a constant temperature of 18–19°C. Larvae were fed a commercial salmonid starter feed (Deng et al. 2003) before the growth trials.

Two 1-week growth trials were conducted to determine the optimum feeding rates of white sturgeon at weeks 6 and 10 after initiation of feeding that corresponded to days 53–59 and 82–88 posthatch, respectively. At the beginning of each trial, sturgeon larvae were captured randomly from the stock tanks and distributed into 24 circular fiberglass tanks with 60 and 25 larvae per trial. Each fiberglass tank (66 cm diameter by 15 cm water depth; 45 L water volume) contained degassed groundwater supplied at 3 L/min per tank. Water temperatures were measured twice daily. Two groups of 20 and 7 larvae, respectively, were captured randomly from the stock tanks for determination of initial body weights and initial body proximate compositions. The larvae were euthanized with an overdose of tricaine methanesulfonate (1 g/L; Argent, Redmond, WA), blotted dry, weighed as a group, placed in plastic bags, frozen in liquid nitrogen, and stored

at −20°C. At the end of each trial, all fishes in a tank were captured, euthanized, pooled, processed, and stored as described previously.

The feed supplied was salmonid soft moist 1/32 W (Rangen, Buhl, ID) with a proximate composition of 16.6% moisture, 48.8% crude protein, 22.3% crude fat, 7.3% ash, and 5.3% NFE. Six feeding rates of 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0% of the body weight per day (BW/d) for the first trial and 0.8, 1.6, 2.4, 3.2, 4.0, and 4.8% BW/d for the second trial were tested in this study. Each feeding rate was assigned randomly to four replicate tanks and the larvae were fed with automatic feeders day and night (Cui et al. 1997). Specific growth rates were calculated using the formula: SGR = (lnWf – lnWi × 100)/*t*, where lnWf is the natural logarithm of the final weight, lnWi is the natural logarithm of the initial weight, and *t* is the time (days) between lnWf and lnWi. Proximate composition (moisture, crude protein, crude fat, and ash) of the initial and final larval body samples and the commercial feed were determined by the AOAC method (Jones 1984). Data were subjected to one-way analysis of variance and optimal feeding rates were determined based on specific growth rates by the broken-line model (Robbins et al. 1979). Statistical analyses were conducted using SAS (SAS Institute, Cary, NC) and tested at $P < 0.05$. Differences between treatment means were determined by Tukey's studentized range test.

31.3 Results

Mortality was very low and unrelated to feeding rates. Broken-line analysis on specific growth rates indicated that the optimum feeding rates of white sturgeon at the 6th and 10th weeks after initiation of feeding were 6.5 ± 0.4 and 3.8 ± 0.1% BW/d, respectively (Fig. 31.1). Growth performances were significantly affected by the feeding rates (Tables 31.1 and 31.2). Final body weights, body weight increases (BWI), and feed efficiencies showed a linear increase, a plateau, or a decrease after the plateau with further increases of feeding rates (Tables 31.1 and 31.2). In both

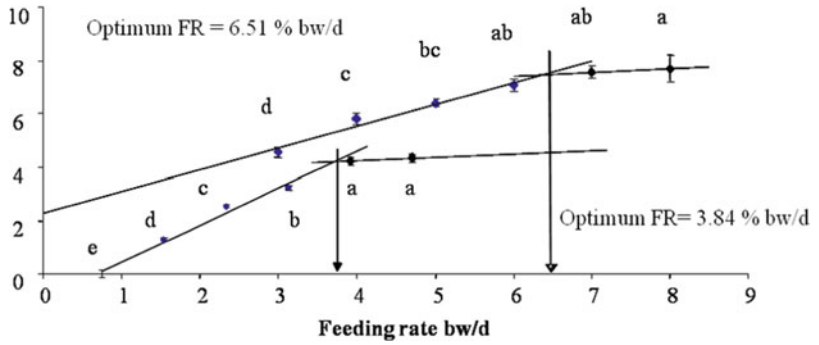

Fig. 31.1 *Broken lines* from 53 to 59 and from 82 to 88 days posthatch

Table 31.1 Growth performances and body proximate composition of sturgeon from 53 to 59 days posthatch

Feeding rate	3.0	4.0	5.0	6.0	7.0	8.0
Final BW (g)	3.83 ± 0.06b	4.27 ± 0.15ab	4.46 ± 0.09a	4.62 ± 0.10a	4.8 ± 0.12 a	4.74 ± 0.20a
BWI (%)	37.5 ± 1.74d	50.2 ± 2.03c	56.6 ± 1.56bc	64.0 ± 2.45ab	69.8 ± 2.69ab	70.9 ± 5.57a
Moisture	84.5 ± 0.06a	84.48 ± 0.02ab	83.98 ± 0.15bc	84 ± 0.11bc	83.7 ± 0.13c	83.6 ± 0.14c
Crude fat	3.42 ± 0.05c	3.74 ±0.07bc	4.02 ± 0.17ab	4.27 ± 0.05a	4.27 ± 0.04a	4.38 ± 0.09a

Initial body weights of sturgeon juveniles was 2.3 ± 0.9 g, and body proximate compositions (%) were: moisture 85.8 ± 0.9, crude protein 8.4 ± 0.6, crude fat 3.4 ± 0.3, and ash 1.4 ± 0.1. Means ± S.E.M. ($n = 4$, $P < 0.05$)

Table 31.2 Growth performances and body proximate composition of sturgeon from 82 to 88 days posthatch

Feeding rate	0.8	1.6	2.4	3.2	4.0	4.8
Final bw (g)	13.3 ± 0.43c	14.27 ± 0.56bc	16.44 ± 0.65ab	17.8 ± 0.54a	17.27 ± 0.43a	18.3 ± 0.64a
BWI (%)	−0.4 ± 0.54e	9.6 ± 1.59d	19.8 ± 1.01c	25.8 ± 0.53b	35.4 ± 1.54a	36.3 ± 1.46a
Moisture	81.7 ± 0.18a	80.93 ±0.16b	80.62 ± 0.2bc	80.1 ± 0.13c	80.4 ± 0.17bc	80.23 ± 0.1bc
Crude fat	4.27 ± 0.21d	4.85 ± 0.12 cd	5.22 ± 0.06bc	5.95 ± 0.05a	5.65 ± 0.22ab	6.16 ± 0.03a

Initial body weights of sturgeon juveniles was 15.78 ± 4.4 g and body proximate compositions (%) were: moisture 83.5 ± 0.2, crude protein 8.5 ± 0.2, crude fat 4.4 ± 0.2, and ash 2.3 ± 0. Means ± S.E.M. ($n = 4$, $P < 0.05$)

trials, body composition was also significantly affected by feeding rates. Body moisture contents showed a general decrease, whereas body lipid contents increased with increasing feeding rates to the optimum, and they remained constant at feeding rates higher than the optimum. On the other hand, ash and body protein contents were not affected by the different feeding rates.

31.4 Discussion

Very low mortality rates were observed (0.05%) in the present study as compared with the mortality rates previously reported for younger (1–4 weeks after initiation of feeding) and smaller (49–366 mg) white sturgeon larvae fed at suboptimum feeding rates (Deng et al. 2003). This could be explained by older fishes (2.8–13.4 g) having a better tolerance of suboptimum feeding than younger and smaller fish. As expected, we observed in both trials a significant reduction of optimum feeding rates with increased age and body weight. The decrease of optimal feeding rates reported between the first and second trials was in agreement with previous investigations. In fact, white sturgeons with body weights between 30 and 100 g had an optimal feeding rate of 2% BW/d (Hung and Lutes 1987). White sturgeon subyearlings with body weights of 250 g had an optimal feeding rate between 1.5 and 2% BW/d (Hung et al. 1989), and yearling specimens with body weights of 750 g had an optimal feeding rate of 1.3% BW/d (Hung et al. 1995). The decrease in body moisture and the increase in body lipid content with increasing

feeding rates up to the optimum rate is a well-established inverse relationship in many fish species (Love 1980).

In conclusion, the optimum feeding rate based on the broken-line analysis of specific growth rates was 6.5 ± 0.4 and 3.8 ± 0.1% BW/d for white sturgeon at weeks 6 and 10, respectively. These values are recommended for use to prevent problems related to over- and underfeeding. Moreover, it would be interesting to examine the modifications of the optimum feeding rate during the period between weeks 6 and 10 after the initiation of feeding.

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Chapter 32
Preliminary Study on the Relationship Between Skin Temperature of Piglets Measured by Infrared Thermography and Environmental Temperature in a Vehicle in Transit

L. Nanni Costa, V. Redaelli, D. Magnani, S. Cafazzo, M. Amadori, E. Razzuoli, M. Verga, and F. Luzi

Abstract During two 14-h journeys, carried out in July and September 2009, respectively, the variation in skin temperature measured by infrared thermography was examined on a total of 12 piglets. A thermal camera was placed in front of the pen during the first journey and above the pen during the second. The temperature inside the vehicle was registered throughout the journeys. A positive linear relationship was observed between skin and internal vehicle temperatures with an R^2 of 0.44 and 0.57 in July and September, respectively. The results obtained in this preliminary experiment showed the possibility of recording thermal images of piglets in transit. Thus, thermography, coupled with other body-temperature-recording techniques, could be valuable for assessing the adaptive efforts of pigs to environmental conditions experienced during transport.

Keywords Pig • Skin temperature • Infrared thermography • Transport

32.1 Introduction

It is well known that the temperature control system of the pig is inefficient in the presence of high environmental temperatures due to a limited sweating capacity and poor heat dissipation. In addition, the physical and psychological stress experienced during transport contributes to increased body temperatures

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(Knowles and Warriss 2007). The thermal state of the pig during transport is particularly difficult to assess because it is dependent on the combined effects of heat and humidity, external environmental conditions, and vehicle ventilation. The measure of body temperatures is particularly relevant to the welfare assessment of pigs during transport, but this parameter is difficult to measure during transit.

Traditional tools used for body-temperature measurements, such as rectal thermometers, require restraining the animal, and can be used only before and after transport. Recently, Mitchell et al. (2005) continuously monitored deep body temperatures during pig transport using a data logger inserted surgically in the peritoneum. This technique is very promising, but it is applicable only on very few subjects who play the role of "sentinels." In the last two decades, infrared thermography (IRT) has been applied to pigs for detecting superficial body temperatures (Stewart et al. 2005). This technique is based on the detection of thermal energy emitted by a body that is converted into an electronic signal that, in turn, is processed by software to produce digital images. Images are produced using a color scale to highlight areas of different temperatures, thus illustrating the thermal gradient of the examined body with an accuracy of 0.1°C. Infrared thermography does not require any contact and is, therefore, a noninvasive technique that can record measurements on subjects that are difficult to reach or approach, or that are in motion. The purpose of this study was to both continuously measure the thermometric profile of piglets during a long journey and evaluate its relationship with the temperature inside the vehicle.

32.2 Materials and Methods

This study was authorized by the Ethical Committee of the University of Bologna. During two 14-h journeys in July and September 2009, skin temperatures were measured by infrared thermography. A total of 12 piglets, six on each trip, were examined. A thermo-camera, Avio TVS 500 (Nec Avio, Dusseldorf, Germany), was placed in front of and above the pens during the first and second journeys, respectively. The camera was set with an emissivity of 0.98 and placed at a distance of 2 m from the subject during the first and 1 m during the second journey. Air temperature calibration was performed automatically by the instrument. On both journeys, the compartment examined was in the front of the deck on the right side of the vehicle. The positioning of the instrumentation required 1.8 m between the floor and the ceiling. Thus, the available volume of the deck was very wide and natural ventilation was not used. Temperature and relative humidity were continuously recorded by a thermo-hygrometer (HOBO Pro v2 loggers, Onset Computer Co., Bourne, USA) located inside the compartment housing the piglets.

The average weight of piglets transported was 10.88 ± 1.97 kg in the first trip and 8.08 ± 0.74 kg in the second. More than 4,000 thermal images were recorded during the journeys and were analyzed using Goratec Thermography Studio Professional software (Goratec Technology, Erding, Germany). The maximum

temperature of each thermal image was considered because the skin of the animals had temperatures much higher than the bedding, walls, and sides of the compartment. The relationship between maximum skin temperatures and the environmental temperatures recorded in the vehicle during the two transports was examined by regression analysis using Proc Reg of SAS (1996).

32.3 Results

Table 32.1 shows the mean values and the measures of variability of the maximum temperatures of the piglets' skin surfaces that were recorded in the vehicle during the journeys of July and September. The different positions of the thermo-camera, shot angles, distances of recording, and body areas made it impossible to directly compare the temperatures recorded in the two journeys. During the second journey, the placement of the camera above the pen led to higher values of skin temperatures and reduced the variability of thermal measurements.

Figure 32.1 shows the results of regression analysis. In both journeys, the relationship between skin temperatures and the temperatures inside the vehicle was linear ($P < 0.001$). The R^2 values were 0.44 and 0.57 in July and September,

Table 32.1 Mean values and measures of variability of environmental temperatures in the vehicle and maximum skin temperatures as measured by thermography

	Mean	S.D.	Minimum	Maximum
July journey				
Temperature inside the vehicle (°C)	26.32	3.79	21.5	35.5
Skin temperature (°C)	35.97	1.12	33.3	38.7
September journey				
Temperature inside the vehicle (°C)	22.27	3.31	16.0	27.7
Skin temperature (°C)	38.13	0.87	36.0	39.9

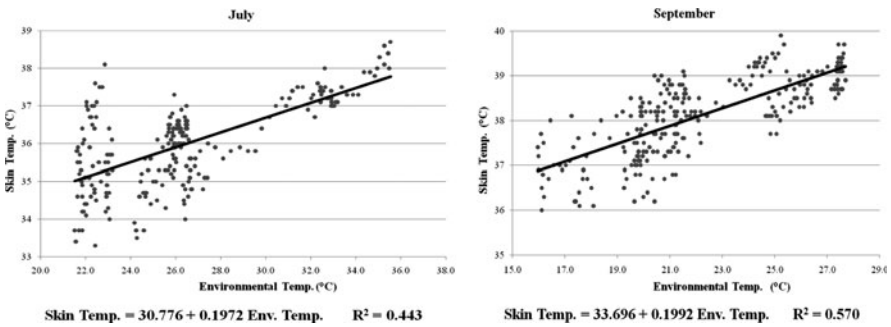

Fig. 32.1 Linear regression between the maximum skin temperature of piglets and the environmental temperature inside the vehicle during the journeys of July and September

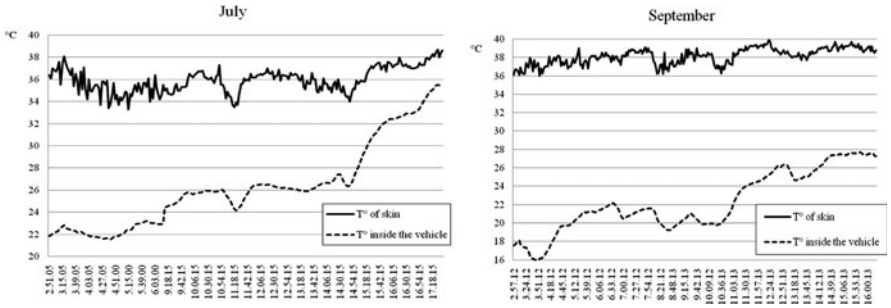

Fig. 32.2 Trends of the maximum skin temperatures and environmental temperatures inside the vehicle during the journeys of July and September

respectively. Data from both the months were tested up to a 5° polynomial regression, which did not increase the R^2 value obtained by the simple regression. It is interesting that the angular coefficients of the two equations were very similar (+0.1972 and +0.1992). Thus, in the range of temperatures recorded during the two transports, an increase of 1°C inside the vehicle resulted in an increase of the maximum skin temperature of 0.2°C. Figure 32.2 illustrates the trends of the maximum skin and internal vehicle temperatures during the two journeys. There was a comparable trend between skin and vehicle temperatures when the vehicle temperatures exceeded 25°C and 20°C during the journeys of July and September, respectively.

32.4 Discussion

To our knowledge, this is the first study using infrared thermography to continuously measure the changes in pig body-surface temperatures during transport. This technique could be applied to controlled transport conditions, even if there are some practical constraints due to the positioning of the camera and the particular environment in which the measurements are performed. Importantly, the vibrations of the vehicle did not influence the quality of the images recorded; however, a loosening of some mechanical parts of the camera occurred. The linear relationship observed between skin and environmental temperatures in the vehicle confirms the results obtained in an environmental chamber by Loughmiller et al. (2001) where, between 10°C and 32°C, this relationship had an R^2 value of 0.97.

The results of this study demonstrate that with infrared thermography it is possible to record the variation of body-surface temperatures and their relationship with the changes of the environmental temperature inside the vehicle. This technique, coupled with deep temperature recording systems, will help to better understand the adaptive efforts of pigs in response to the environmental conditions experienced during transport and, specifically, extreme temperatures.

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Chapter 33
Influence of Sample Storage on the Quality of DNA Extracted from Milk of Goats Fed Conventional or Transgenic Soybean

R. Tudisco, S. Calabrò, M.I. Cutrignelli, M. Grossi, V. Piccolo, and F. Infascelli

Abstract PCR approach was used in order to investigate the presence of plant DNA in pellet of somatic cells from fresh or stored milk of twenty grazing goats fed concentrate (18% CP; 1.03 UFL/kg, as fed) containing conventional or genetically modified soybean meal. The quantity and the purity of DNAs extracted from fresh samples were higher than those of samples stored at −20°C (ng/μl: 51.6 *vs* 20.8; A_{260}/A_{280}: 1.7 *vs* 1.4). The frequency of chloroplast sequence, for both groups, ranged between 70 and 100% in fresh and between 30 and 40% in stored samples. The frequency percentage of specific soybean gene was lower in stored (25–33%) than in fresh samples (56–88%). Transgenic fragments were found only in milk from goats fed GM soybean. For 35S promoter, the frequency was 38–75% in fresh and 25% in stored samples, while for CP4 EPSPS, it was 38–50% in fresh and 25% in stored samples.

Keywords Milk • Goat • DNA extraction • Transgene

Abbreviations

CP	Crude protein
DM	Dry matter
GM	Genetically modified
s.e.	Solvent extract

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33.1 Introduction

The use of PCR is gaining large acceptance as a reliable diagnostic approach for food safety and traceability (Mafra et al. 2008), as also demonstrated by several studies on detection of plant DNA in animal organisms (EFSA 2008). Fragments of nonspecific, high-copy number gene and specific maize and soybean single copy genes (Einspanier et al. 2001; Phipps et al. 2003; Poms et al. 2003; Rizzi et al. 2008; Tudisco et al. 2010) as well as transgenic sequences (Tudisco et al. 2010) were detected in milk. Several potential PCR inhibitors (fat, protein, enzyme, etc.) have been described in milk, which can interfere with the quality of extracted DNA (Rossen et al. 1992). In the present trial, we evaluated the influence of sample storage on quality, purity, and amplification of feed DNA from milk of goats fed conventional or GM soybean.

33.2 Materials and Methods

The trial was carried out on a farm in Casaletto Spartano (province of Salerno) where 20 pluriparous, grazing goats were equally divided into two homogeneous groups (A and B) based on number of calving and previous milk yield. Animals were fed concentrate (18% CP; 1.03 UFL/kg, as fed) containing conventional (group A) or GM (group B) soybean s.e. meal. GM soybean (Roundup Ready®, RR) has been modified with the *EPSPS* gene (encoding 5-enolpyruvilshikimate-3-phosphate synthase protein) from the CP4 strain of *Agrobacterium tumefaciens*, which confers tolerance to the glyphosate family of herbicides. Twenty days after delivery and every 15 days, five individual milk samples (100 ml) were collected, immediately transported into the laboratory by fridge bag, and incubated overnight at 4°C. The day after, each sample was skimmed and divided into six aliquots (15 ml each) that were centrifuged for 20 min at 2,000 × *g* at 4°C to obtain a pellet of somatic cells. This pellet was twice washed with PBS (1×, pH 7.2, with 0.5 mM EDTA). DNA from three pellets was immediately extracted according to the Wizard method (Promega); concentration and quality were determined by spectrophotometry at 260 and 280 nm (BioPhotometer, Eppendorf). The other three pellets were stored at −20°C and subsequently analyzed. The amplification and quality of DNA (three replicates per aliquot) were assessed by endpoint PCR (Gene Amp PCR System 2400, Applied Biosystems) using the Cap144/469 primers (Bottero et al. 2003) to amplify a conserved portion (326 bp) of caprine mtDNA, which encodes the 12S ribosomal RNA (12S rRNA) gene of mitochondrial DNA. In milk samples and in conventional and transgenic soybean s.e. meal, as plant control, a fragment of the high-copy number chloroplast gene (*trnL*, 100 bp) was searched (Terzi et al. 2004). Finally, species-specific primers for conventional and GM soybean were used: Le1 5/3, which amplifies the soybean lectin gene (Kuribara et al. 2002); 35S 1/2 and CP4 EPSPS 1/2, which amplify, respectively, part of the 35S promoter (Lipp et al. 1999) derived from the cauliflower mosaic virus and part of the specific gene sequence

(CP4 EPSPS) (Hernández et al. 2003). PCR was performed three times per aliquot, and samples with positive results at least twice were judged as positive (Chowdhury et al. 2003). The identity of amplification was verified by the ABI PRISM® 310 Genetic Analyzer (Applied Biosystems) and confirmed by comparison with all sequences in the international nucleotide nonredundant databank.

33.3 Results

The quantity and purity of DNA extracted from fresh samples were higher than samples stored at −20°C (ng/µl: 51.6 vs. 20.8; A_{260}/A_{280}: 1.7 vs. 1.4). The frequency of the chloroplast sequence, for both groups, ranged between 70 and 100% in fresh samples and between 30 and 40% in stored samples (Fig. 33.1a). In the samples positive for the chloroplast sequence for both groups, the fragments of specific soybean gene were detected (56–88% in fresh and 25–33% in stored

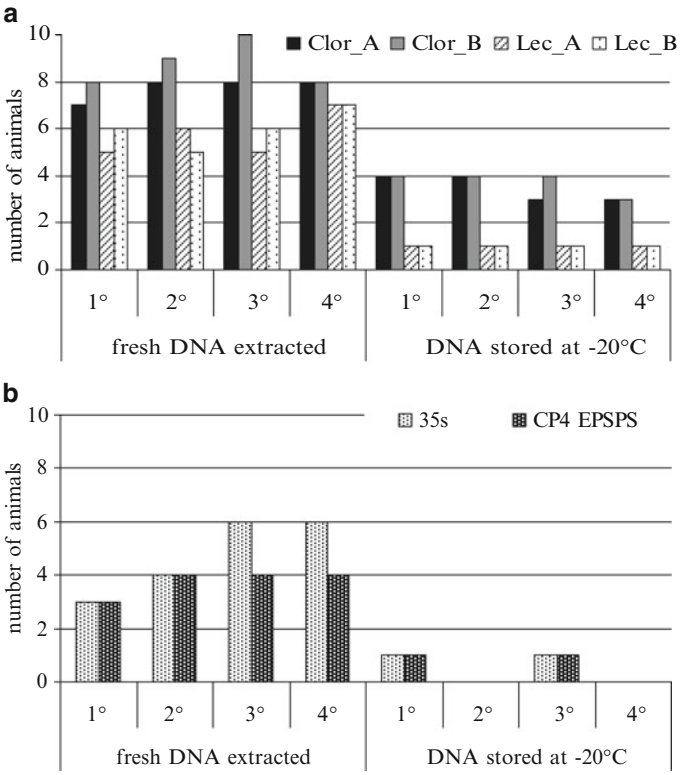

Fig. 33.1 Detection of chloroplast gene (clor, a), soybean lectin gene (lec, a), 35S promoter (35S, b), and CP4 EPSPS gene (CP4, b) fragments in milk samples from goats fed conventional (**a**) or transgenic (**b**) soybean s.e. meal

samples, Fig. 33.1a). Transgenic fragments were found only in milk from goats fed GM soybean. In the case of the 35S promoter, the frequency was 38–75% in fresh samples and 25% in stored samples; CP4 EPSPS were amplified in 38–50% of fresh samples and 25% of stored samples (Fig. 33.1b).

33.4 Discussion

According to other authors (Einspanier et al. 2001; Phipps et al. 2003; Poms et al. 2003; Tudisco et al. 2010), it has been confirmed that the detection of specific gene fragments is more difficult than that of nonspecific fragments. In addition, our results confirm the possibility to detect transgenic fragments in milk samples from animals fed GM feed, as previously reported (Tudisco et al. 2010). Concerning food traceability, the influence of sample storage on the concentration as well as on the quality of DNA extracted has been demonstrated; indeed, both parameters were decreased in the pellet of somatic cells stored at −20°C.

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