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RESEARCH PROJECT:

**DIFFERENTIAL DIAGNOSIS AND PATHOGENESIS OF NON INFECTIOUS, NODULAR
TO DIFFUSE, GRANULOMATOUS *and* PYOGRANULOMATOUS SKIN LESIONS IN
DOGS**

**(PATOLOGIE CUTANEE NON INFETTIVE A CARATTERE NODULARE E DIFFUSO DI
TIPO GRANULOMATOSO E PIOGRANULOMATOSO NEL CANE: DIAGNOSTICA
DIFFERENZIALE E PATOGENESI)**

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Introduction

Non infectious, non neoplastic, nodular to diffuse, granulomatous/pyogranulomatous skin lesions are rarely reported in dogs and they often represent a clinical and histological diagnostic challenge (1,2). However, a precise diagnosis is pivotal for their adequate approach and treatment that differs for each entity. Canine non infectious granulomatous/pyogranulomatous (G/PG) skin lesions are mainly represented by sterile pyogranuloma/granuloma syndrome (SPGS), nodular sterile panniculitis (NSP), juvenile sterile granulomatous dermatitis and lymphadenitis, reactive histiocytosis (RI), sterile sarcoidal granulomas and xanthomatosis (1,2,3).

SPGS is an idiopathic canine skin disorder, characterized clinically by nodules or plaques and histopathologically by multifocal, nodular to diffuse, pyogranulomatous/granulomatous dermal inflammatory infiltrates. Early microscopical lesions are represented by elongated, vertically oriented periadnexal, pyogranulomas to granulomas with a “sausage” shaped appearance. In later stages, the inflammatory infiltrate coalesces into a more diffuse inflammation that makes SPGS very difficult to differentiate from other diseases (3,4). Diagnosis of SPGS is based on clinical appearance, histopathological findings and lack of microorganisms after special stain application (PAS, Ziehl-Neelsen and Gram), failure to demonstrate foreign bodies by polarized light microscopy, and negative microbiological tissue cultures (3,4). The aetiology of SPGS is currently unknown; however, it has been hypothesized that SPGS may be related to an excessive immune response against persistent endogenous or exogenous antigens, causing a persistent granulomatous inflammatory reaction. Recently some reports have described the presence of *Leishmania* spp. antigens and amastigotes in previously erroneously diagnosed SPGS (3,5,6). *Leishmania* organisms can be detected by immunohistochemistry on skin biopsies with histological lesions suggestive of SPGS, in which no microorganisms have been viewed either on haematoxylin and eosin (H&E) or on specially stained sections. Canine Leishmaniosis (CL) is characterized by a plethora of

cutaneous histopathological lesions that may mimic various diseases including SPGS and, thus, if only very small numbers of amastigotes are present, the histopathological differential diagnosis between the two diseases may be difficult (7,8). Also, if only residual antigens persist in skin lesions, amastigotes are not present thus, histology is insufficient to detect the cause of the inflammation. In such cases, immunohistochemical and/or polymerase chain reaction (PCR) techniques are required to rule out definitively the presence of *Leishmania* organisms or persistent antigens (9-12). Interestingly, in humans, mycobacteria and other microorganisms have been detected by PCR in granulomatous diseases classically reported as sterile, such as sarcoidosis (13,14). Some veterinary studies have tried to detect this organism in canine skin samples, diagnosed as sterile, with negative results (6). *Leishmania* spp., *Mycobacterium* spp. and other infective partially acid fast organisms (e.g. *Nocardia* spp, *Actinomyces* spp and *Serratia* spp) might represent a possible source of persistent exogenous antigenic stimulation in cases of canine SPGS.

Aims of the study

The aims of the current research project were:

- To select retrospectively and prospectively skin samples with a histopathological diagnosis of cutaneous sterile nodular lesion.
- To investigate the presence/absence of microorganisms by haematoxylin and eosin, special stains as PAS, Ziehl–Neelsen, Gram and Fite Faraco.
- To exclude presence of foreign bodies with polarized light examination.
- To detect the presence/absence of *Leishmania* spp. antigenic persistence in canine skin samples diagnosed histologically as sterile nodular lesions (e.g. SPGS) by immunohistochemistry.
- To perform identification of *Leishmania* spp., *Mycobacterium* spp., *Serratia marcescens* and *Nocardia* spp. by means of Real Time-PCR in canine skin samples diagnosed histologically as sterile nodular lesions.
- To evaluate the effectiveness of RT-PCR technique in detecting *Leishmania* organisms in comparison with immunohistochemistry (Anti-*Leishmania*, anti-Bacillus Calmette-Guerin).
- To identify cases of SPGS among samples with a previous diagnosis of sterile granulomatous nodular skin lesions.

Non infectious, nodular to diffuse, granulomatous *and* pyogranulomatous nodular skin lesions: aetiology, clinical manifestations and differential diagnosis

In dogs, skin lesions with a histological diagnosis of granulomatous dermatitis are different and numerous. Furthermore they may have different aetiology. Granulomatous nodular dermatitis with granulomatous *or* pyogranulomatous, nodular to diffuse infiltrates, are generally divided in two groups: infectious and non-infectious diseases (2,7,15).

Commonly, they are sub-divided in four sub-groups: infections with detectable agents, infections with no detectable agents, not infective lesion with detectable etiologic agent (e.g. foreign body); not infective lesion with immune-mediated aetiology (e.g. sterile idiopathic dermatitis) (16). The presence of pathogens easily induces pyogranulomatous skin lesions and histopathological exams give the correct diagnosis using special stains, immunofluorescence, etc. On the other hand, some infective agents as *Leishmania* spp. and *Mycobacterium* spp. may be no detectable with special stains; in these cases PCR and immunohistochemistry may be necessary for a correct diagnosis (16). In more difficult cases, clinical examination and many different laboratory tools are necessary to reach the diagnosis. Nodular sterile dermatitis has to be differentiating from infectious nodular dermatitis for a correct approach to the patients. Skin lesions may be clinically represented by: single papules, nodules and/or plaques, localized or diffuse, alopecic or haired, with different sizes (17). Unfortunately, clinical lesions are not suggestive of the aetiology and may lead to several differential diagnoses.

In dogs, nodular sterile dermatites are a group of skin diseases of unknown origin (1,2,8). They include sterile pyogranuloma/granuloma syndrome, nodular sterile panniculitis, juvenile sterile granulomatous dermatitis and lymphadenitis, reactive histiocytosis, sterile sarcoidal granulomas and xanthomatosis (5,16). The diagnosis is reach through clinical history, skin lesions, exclusion of

foreign bodies with polarized light, exclusion of infective agents with special stains, bacterial or mycological culture (4). The exclusion and/or detection of infective agents are mandatory for the adequate treatment of the patient. A wrong diagnosis of “sterile dermatitis” may lead to an immunosuppressive systemic therapy that may be fatal in infectious cases (e.g. Leishmaniosis).

Sterile pyogranuloma/granuloma syndrome

Sterile pyogranuloma/granuloma syndrome (SPGS) is uncommon disease in dogs (3). There is no sex or breed predisposition, but most cases are reported in male dogs and particularly in Great Danes, Dachshunds, Boxer, English bulldog, Collie, Miniature Doberman Pinscher, Golden Retriever and Weimaraner (2,17). The histiocytic nature of the inflammation, coupled with the good response to glucocorticoids and/or other immunosuppressive drugs, suggests the likelihood of immune dysfunction, perhaps associated with persistent antigenic drive (3). Skin lesions are represented by multiple, firm, well-demarcated, variably alopecic dermal plaques or nodules (between 0.5 and 2 cm in diameter), generally asymptomatic. Secondary bacterial infection may be present in ulcerated nodules (figure 1, 2). In the majority of cases the skin lesions are detectable on the head (dorsal muzzle, pinnae and periorbital areas) and distal limbs, but they may occur even on the trunk and neck (2,3,17); recently a case with tongue insolent has been reported (18). No systemic signs have been reported in SPGS even if a moderate regional lymphadenopathy has been detected (17); in rare cases not malignant hypocalcaemia has been reported (19).



Figure 1: nodular skin lesions with hyperpigmentation and ulceration in a Labrador retriever with SPGS



Figure 2: alopecia, erythema and ulcerated nodular-papular lesions in a Beagle with SPGS

In SPGS histological pattern is mainly represented by granulomatous vs pyogranulomatous multifocal, nodular to diffuse dermatitis (4). The epidermis is normal to moderate acanthotic with occasional ulcerations. Granulomatous and/or pyogranulomatous inflammation, with discrete to confluent granulomas and/or pyogranulomas extends from the dermis to the panniculus; sometime it extends to the deeper subcutis (3). Early inflammation is most prominent perivascular and may be strictly oriented along adnexa, sometimes sausage-shaped or nodular pattern. In more severe forms the infiltrate is diffuse to the dermis. Inflammatory cells are mainly represented by macrophages, lymphocytes and neutrophils in association with plasma cells and giant cells (3,4). More severe cases, differentiation from late stage of reactive histiocytosis can be difficult as there can be increased infiltration of macrophages, neutrophils secondary to ischemic necrosis (3).

Juvenile sterile granulomatous dermatitis and lymphadenitis

This skin disease is generally known as juvenile cellulitis (JC) and the diagnosis is mainly clinical based on history, age of onset, topographic localization of skin lesions and clinical aspects (2,3). Juvenile cellulitis is usually seen in dogs older than 3 weeks but less than 4 months (3), even if a case has been reported in a 2 years old dog (20). No sex predisposition is reported but breed predilections probably exist for Golden Retrievers, Dachshunds, Gordon Setters, Labrador Retrievers and Lhasa Apsos (3). The onset of the disease is dramatically acute with swelling on the face. Clinical lesions are generally symmetrical and they are mainly localized on the muzzle, periorbital region, chin and pinnae. Erythema, papules, pustules, nodules, alopecia and finally crusts represent the skin lesions. Fistulae may be present in late stage of the disease were secondary bacterial infections are present. Systemic signs as fever, anorexia and joint pain are detectable (2,3,15). Histopathology of early lesions is mandatory for definitive diagnosis. In older skin lesions, may be difficult to differentiate from other granulomatous diseases. The epidermis may be variably normal, acanthotic or ulcerated in more severe cases with marked exudation. The dermal lesions are

multiple discrete to confluent granulomas and pyogranulomas comprising nodular clusters of large epithelioid macrophages with variably sized cores of neutrophils; these ones are often at least partly perifollicular with some discretely perifollicular at the level of isthmus (3). Sebaceous glands are often obliterated and pyogranulomas may extend to the panniculus (3). Chronic lesions may be scarred with prominent interlobular pannicular fibrosis.

Canine Sarcoidosis

Canine sarcoidosis is an extremely rare and idiopathic disease and only few cases have been reported (21). Case numbers are too small to infer sex and/or breed predilections, even if reported cases were in Shetland Sheepdogs (21). Clinical lesions are represented by papules, plaques and nodules mainly localized on the trunk, neck, face and ears. Some authors include canine sarcoidosis into SPGS as a sub-group of this disease (2,3). Histological lesions are characterized by multifocal “naked” granulomas. These ones are composed mainly by epithelioid macrophages without a peripheral cuff of lymphocytes. Peripheral palisating is absent and granulomas are oriented within the superficial to deep dermis; subcutaneous involvement has been reported.

Reactive Histiocytosis

Reactive histiocytosis (RI) is a quite uncommon and poorly understood disease in dogs. The epidemiology of RI is controversial as the disease. Some authors reported a breed predilection for Collies and Shetland Sheepdogs, while others suggest an over-representation of Bernese Mountain dog, Rottweiler, Golden Retrievers, Labradors Retrievers and Irish Wolfhounds (3). The age of onset ranges from 2 to 11 years, while a sex predisposition has not been reported. As for many of the previous described “sterile” skin nodular diseases, the aetiology and pathogenesis of RI are unknown. Skin lesions are mainly characterized by multiple, non pruritic and non painful, haired or partially alopecic, cutaneous nodules and plaques. They are mainly detectable on the head, neck,

extremities, perineum and scrotum (3). Bacterial infections may be present in ulcerated skin lesions. Lesions are consistent with a reactive inflammatory process in response to persistent antigen, which remains unknown. The epidermis is variably normal or ulcerated; there is a nodular to diffuse infiltrate from the middle dermis to the superficial subcutis, often referred as a “bottom-heavy” infiltrate. This is composed of mainly histiocytes, small lymphocytes and neutrophils. The histiocytes have a large, round to oval, indented or folded and twisted, vesicular nuclei. Small lymphocytes may contribute up to 50% of the infiltrating population, while neutrophils vary and increase in necrosis. Angiocentric nodules characterized the RI. Unfortunately, some cases of RI may mimic SPGS. In these cases immunocytochemistry and immunohistochemistry are mandatory for the definitive diagnosis. Histiocytes are CD45, CD18, CD1c, CD11c, CD90, CD4 and MHCII positive, E-cadherin negative (3,22).

Materials and methods

Sample selection

Forty formalin-fixed, paraffin-embedded skin biopsies were collected from 40 dogs through the Diagnostic Pathology Service of the Veterinary Faculty of Milan (UNIMI, Italy) and through the Pathology Service of Ambulatorio Veterinario Associato (AVA, Turin, Italy). All samples had lesions corresponding to the sterile granulomatous/pyogranulomatous pattern, characterized by multifocal, nodular to diffuse, periadnexal and perifollicular pyogranulomas/granulomas, in which neither microorganisms nor foreign bodies were detected by H&E and polarized light. Special stains (PAS, Ziehl–Neelsen and Gram) were negative for infective agents.

The samples collected during the three years were stained with a Fite Faraco kit [Diapath™ SPA, Martinengo (BG), Italy] too. They all resulted negative for mycobacteria, confirming the previous absence of micro-organisms within the selected histological sections.

All the samples, collected between 2011 and 2013, that were negative for all special stains and where neither microorganisms nor foreign bodies were detected, were examined by RT-PCR analysis for *Leishmania* spp., *Mycobacterium* spp., *Serratia marcescens* and *Nocardia* spp. Furthermore, immunohistochemistry (IHC) was performed for *Leishmania* spp.

Immunohistochemical technique to detect Leishmania organisms

Sections were deparaffinized, rehydrated and incubated for 30 min in a solution of 200 ml TBS+ 2 ml H₂O₂ at 36% plus 2.5-3 mg sodium azide to quench endogenous peroxidase. Sections were then washed with tris buffer solution (TBS). Antigen retrieval was obtained by heating treatment of the samples in a Citrate buffer solution (pH 6.4) 1 minute with Microwave at maximum power and 3+3

minutes at 750 W. Samples were cooled at room temperature and rinsed with TBS and then incubated with normal horse serum for 30 minutes at room temperature for blocking of unspecific binding sites. Serum was removed and mouse monoclonal anti-Leishmania primary antibody was applied at a 1:6500 dilution. Samples were incubated overnight (12 hours) at 4° C and subsequently washed with TBS. A biotinylated anti-mouse antibody (H+L, Dako™ Corp., Glostrup, Denmark) at a 1:400 dilution was added and sections were incubated for 30 minutes. After three 5-min rinses in TBS, tissue sections were incubated with avidin-biotin complex (ABC kit, Vector™ Laboratories, Burlingame, CA, USA) for 30 minutes at room temperature and then washed with TBS. The sections were then incubated with the AEC chromogen kit (Vector™ Laboratories, Burlingame, CA, USA) rinsed in tap water, counterstained with Mayer's haematoxylin, dehydrated and mounted with glycerine.

Immunohistochemical technique for Bacillus Calmette-Guérin (BCG) stain

Sections were deparaffinized and rehydrated. Inhibition of endogenous peroxidase was performed incubating slides for 30 min in a solution of 200 ml TBS+ 2ml H₂O₂ at 36% plus 2.5-3 mg sodium azide to quench endogenous peroxidase. Sections were then washed with TRIS buffer solution (TBS) for 5 minutes. Antigen retrieval was obtained by heating treatment of the samples in a Citrate buffer solution (pH 6.4); they were boiled into a microwave oven at maximum heat for 1 min, then 2 times at 750 watt for 3 min each time. Sections were cooled at room temperature. Samples were incubated with blocking solution 1 ml TRIS plus 15 µl goat serum for 30 min; they were subsequently washed with TRIS buffer solution for 3 rinses of 5 minutes each. The polyclonal primary antibody anti-Bacillus Calmette-Guérin made in rabbit was applied to the samples at a 1/5000 dilutions and leaved into a humid chamber for overnight at 4° C. After washing in a TRIS solution three times for 5 min, each section was incubated with a biotinylated anti-rabbit antibody

(H+L, Dako™ Corp., Glostrup, Denmark) for 30 minutes. Samples were washed with TRIS buffer solution for 5 min, incubated with 100 µl avidin-biotin complex (ABC) for 30 min at room temperature and then washed again with TRIS buffer solution for 5 min. The sections were then incubated with acetate buffer for 5 min and the AEC chromogen kit (Vector™ Laboratories, Burlingame, CA, USA) and the reaction was monitored. Finally they were counterstained with Mayer's haematoxylin for 1,5 min, rinsed in tap water, counterstained with Mayer's haematoxylin, dehydrated and mounted with glycerine (23).

Real time molecular biology technique (RT-PCR) to detect Leishmania spp., Mycobacterium spp., Serratia marcescens and Nocardia spp.

DNA extraction

A commercial kit QIAamp FFPE Tissue® (Qiagen™, Milan, Italy) for purifying DNA from formalin-fixed, paraffin-embedded tissue sections was used. The kit uses special lysis conditions to release DNA from tissue sections and to overcome inhibitory effects caused by formalin cross linking of nucleic acids. Sections were placed in microcentrifuge tube and deparaffinized with xylene, dried and re-suspended in 180 µL of tissue digestion solution containing 20 µL of proteinase K. Samples were incubated for 1 hour at 56° C and at 90° C for another one. After centrifugation samples were added to 200 µL of buffer solution and then of ethanol (96-100%). The lysate was transferred into the column with resin and centrifugated at 6000 g for 1 min. At the end the extract was suspended into 50 µL of buffer TLE (Tris Low EDTA) DNase-free and stored at -20° C until use for PCR reaction.

Primers selection

<u>TARGET</u>	<u>SET</u> <u>PRIMERS</u>	<u>SEQUENCE</u>	<u>BP</u>	<u>REF</u>
<i>Leishmania spp</i>	Liesh- 13a	5'-GTGGGGGAGGGGCGTTCT-3'		(24)
	Leish-13b	5'-ATTTTACACCAACCCCAAGTT-3'	117	
<i>Mycobacterium</i> spp	ITS-F	5'-TGGATCCGACGAAGTCGTAACAAGG-3'		(25)
	mycom-2	5'-TGGATAGTGGTTGCGAGCAT-3'	270- 400	
<i>Serratia</i> <i>marcescens</i>	SMSF	5'-GGTGAGCTTAATACGTTTCATCAATTG-3'		(26)
	SMSR	5'-GCAGTTCACAGGTTGAGCC-3'	179	
<i>Nocardia spp</i>	NG1	5'-ACCGACCACAAGGGGG-3'		(27)
	NG2	5'-GGTTGTAAACCTCTTTTCGA-3'	590	

Real-time PCR

The real-time PCR reactions were carried out in tubes in volume of 25 µl. The PCR assays were performed on StepOne™ (Life Technologies, Monza, Italia) that is a real time machine that allows both rapid PCR cycling and continuous monitoring of product formation (28). The formation of double-stranded PCR products is detected by SYBR Green, an intercalating dye, that fluoresces strongly when bound to double-stranded DNA, so when PCR products are formed, an increase in fluorescence is observed (29,30). After PCR amplification, the StepOne™ machine can monitor melting of the DNA with increasing temperature by measuring the decrease in fluorescence as SYBR Green I is released. For convenience, the negative derivative of fluorescence versus

temperature is plotted to give a discrete melting peak. When the melting temperature of the PCR products is analyzed in this way, it is not necessary to visualize the PCR products on an agarose gel.

Leishmania spp. and Mycobacterium spp.

The final PCR mixture contained 2,5 µl each of forward and reverse primers (100 mM), 15 µl of Fast SYBR™ Green (Life Technologies Italia, Monza), and 2 µl of DNA. Real-time PCR cycling profile consisted of 95° C for 2 sec, which was followed by 40 cycles of denaturation at 95° C for 0,01 sec and annealing at 60° C for 20 sec. After amplification, a melting step was performed, consisting of 95° C for 15 sec, cooling to 60° C for 1 min and finally a slow rise in the temperature to 95° C at a rate of 0,3 ° C 15 sec with continuous acquisition of fluorescence decline.

Serratia marcescens

The real-time PCR mixture contained 5 µl template DNA, 2 µl forward and reverse primers (100mM each), and 2 µl Fast SYBR™ Green (Life Technologies Italia, Monza). PCR amplification comprised an initial denaturation cycle at 95° C for 10 min, followed by 45 amplification cycles consisting of 95° C for 15 sec, annealing at 60° C for 1 min. After amplification, a melting step was performed, consisting of 95° C for 0 sec, cooling to 60° C for 1 min and finally a slow rise in the temperature to 95° C at a rate of 0,1° C 21 sec with continuous acquisition of fluorescence decline.

Nocardia spp.

The real-time PCR mixture contained 2µl template DNA, 2µl forward and reverse primers (1 mM each), and 2 µl Fast SYBR™ Green (Life Technologies Italia, Monza). PCR amplification comprised an initial denaturation cycle at 95° C for 10 min, followed by 40 amplification cycles consisting of 95° C for 10 sec, annealing at 55° C for 10 sec, and extension at 72° C for 60 sec. After amplification, a melting step was performed, consisting of 95° C for 0 sec, cooling to 65° C for 60 sec and finally a slow rise in the temperature to 95° C at a rate of 0,1° C 21 sec with continuous acquisition of fluorescence decline.

Results

Breeds, age and sex are resumed in table 1 and figure 3. Dermatological lesions were localized on the head in 22/40, on the trunk in 9/40, on the legs in 4/40, on the neck in 1/40, on the prepuce in 1/40; in 3/40 they were diffuse (figure 4). Skin lesions were represented by nodules in 25/40, alopecia in 7/40, exfoliation and ulcers 3/40, crusts in 2/40; in 3/40 cases they were not reported (figure 5). Special histochemical stains (PAS, Ziehl–Neelsen, Gram and Fite-Faraco) were negative for infective agents and polarized light failed to reveal any foreign bodies.

The histological pattern was nodular to diffuse in 17/40, focally nodular in 12/40 and diffuse in 11/40 (figure 6). The infiltrate was granulomatous in 20/40, pyogranulomatous in 8/40 and granulomatous/pyogranulomatous in 12/40 (figure 7).

The histological diagnosis was SPGS in 14/40, granulomatous vs pyogranulomatous dermatitis in 15/40, sterile nodular panniculitis in 4/40, reactive histiocytosis in 5/40 and juvenile cellulitis in 2/40 (figure 8, 9).

BCG was negative for infective agents. Immunohistochemistry for *Leishmania* spp. was positive in 1/40 samples collected retrospectively (figure 10) while RT-PCR for *Leishmania* spp. was positive in 4/40 biopsies collected (figure 11).

RT-PCR for *Serratia marcescens* was positive in 2/40 samples (figure 12), while RT-PCR for *Mycobacterium* spp. and *Nocardia* spp. were negative in all 40 samples.

Table 1: summary of cases included during PhD (years 2011-2013)

Breeds	1 American Staffordshire, 1 Boxer, 1 Epanol Breton, 1 Pit bull, 1 Rottweiler, 1 Poodle, 2 Dachshund, 2 Bernese dogs, 2 Jack Russell Terrier, 2 English Setter, 3 Beagle, 5 German Shepherd, 6 Labrador retriever, 12 Mixed breed
Sex	18 male, 12 female and 10 neutered female
Age	Between 1 and 12 years

Figure 3: Histological samples were obtained from different breeds as showed in the picture

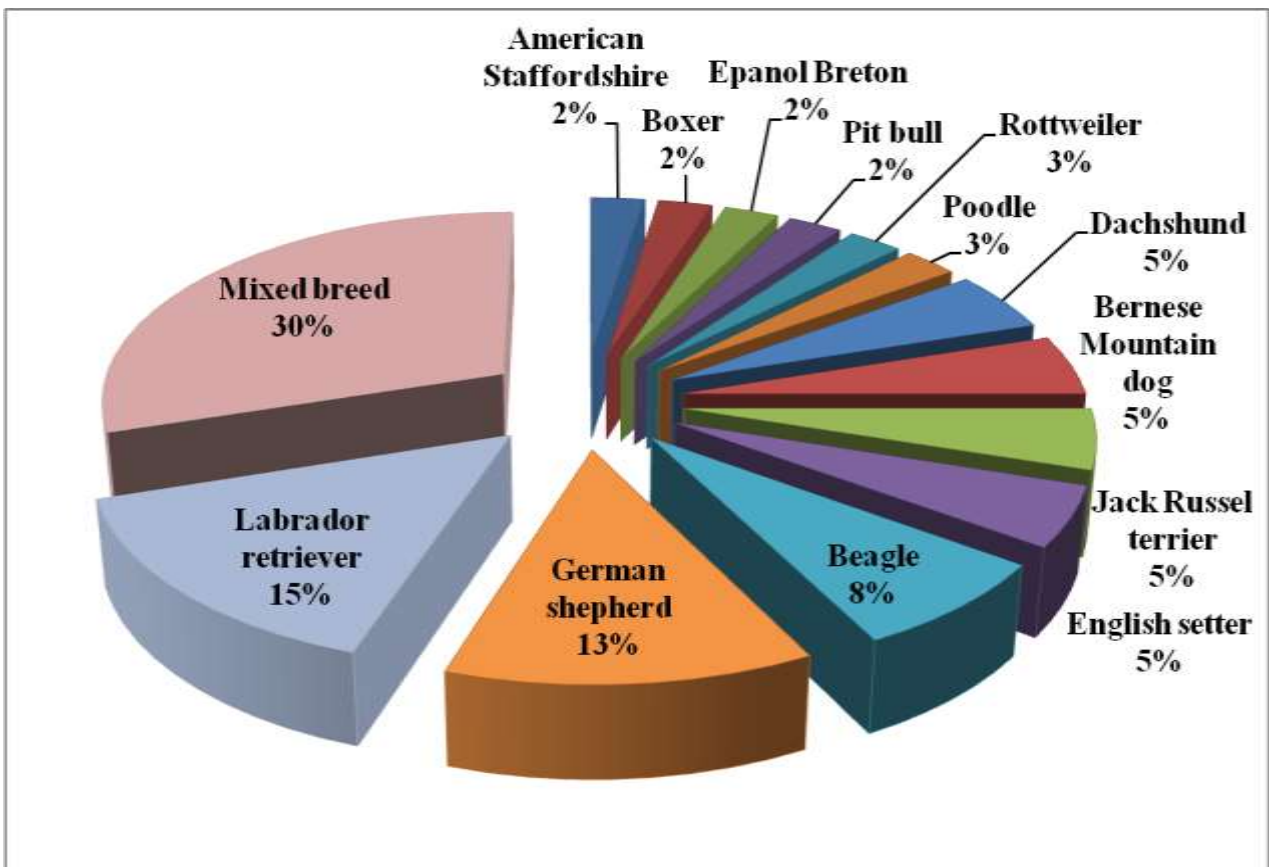


Figure 4: localization of skin lesions on the body surface.

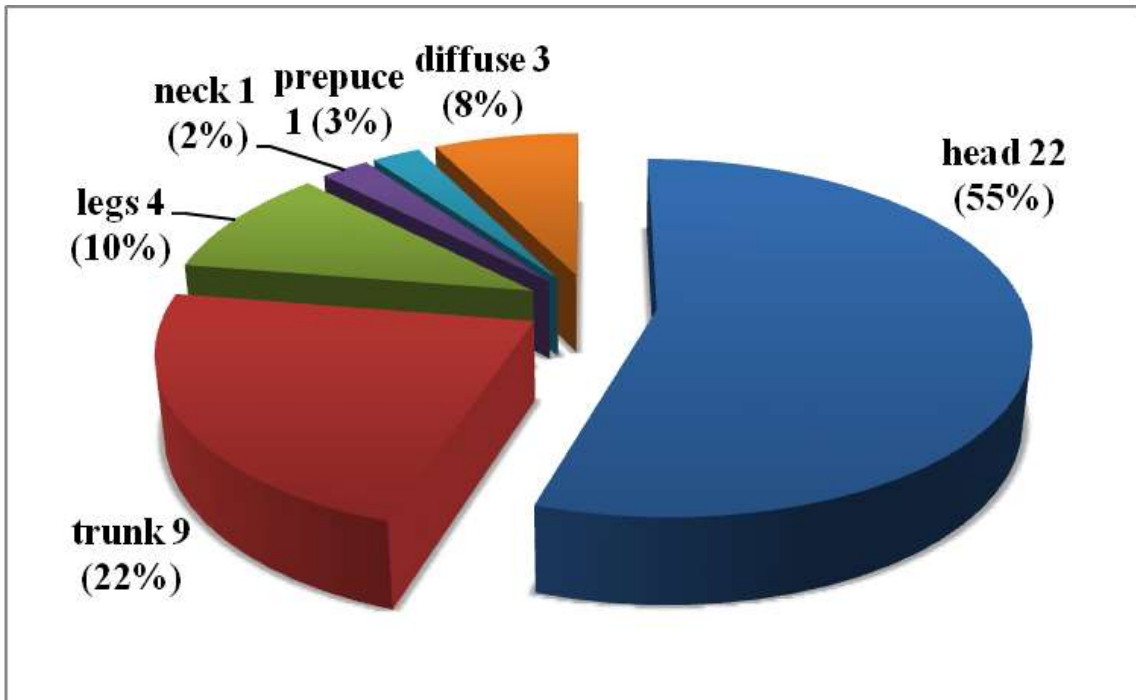


Figure 5: type of skin lesions identified from which the skin biopsies were taken

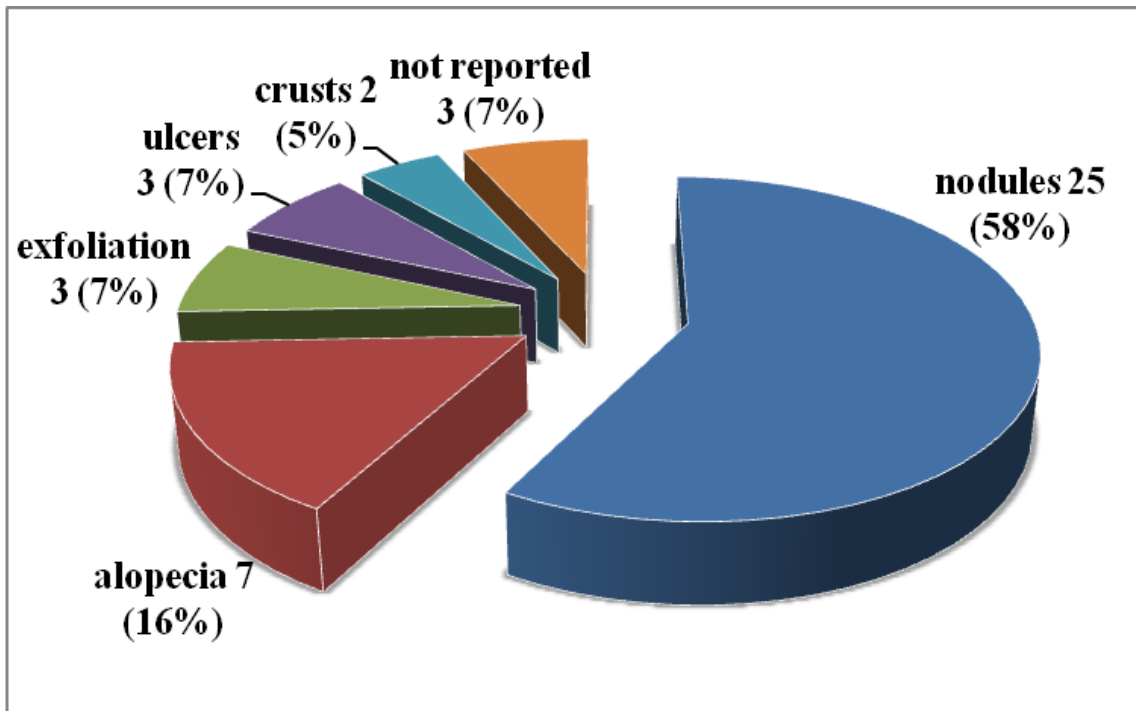


Figure 6: type of pattern identified in the selected skin samples

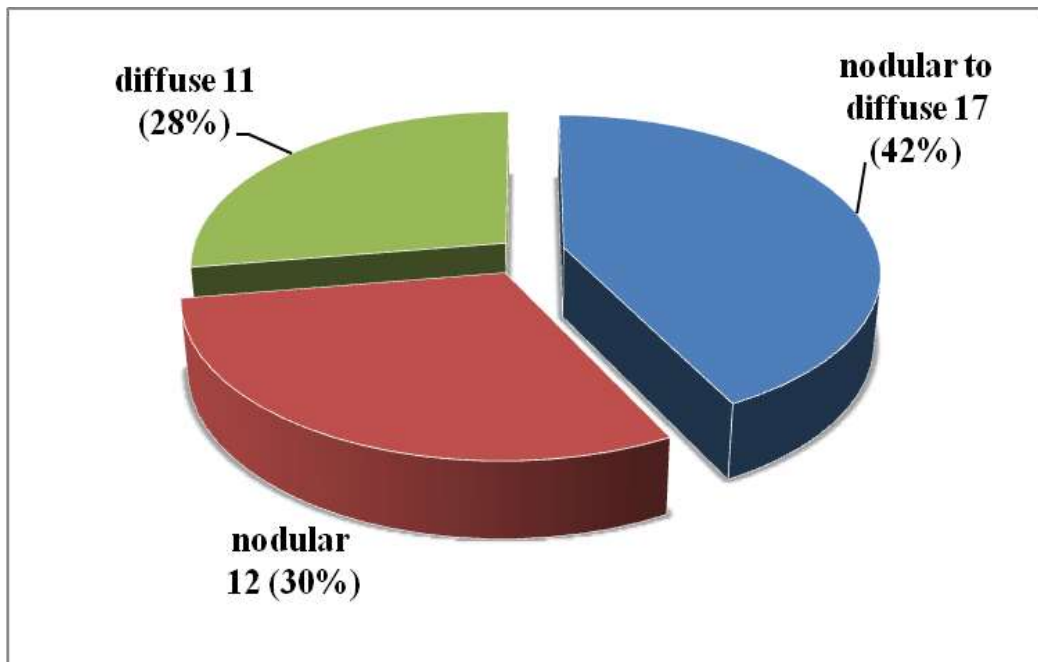


Figure 7: infiltrate identified in all skin biopsies (G: granulomatous; P: pyogranulomatous)

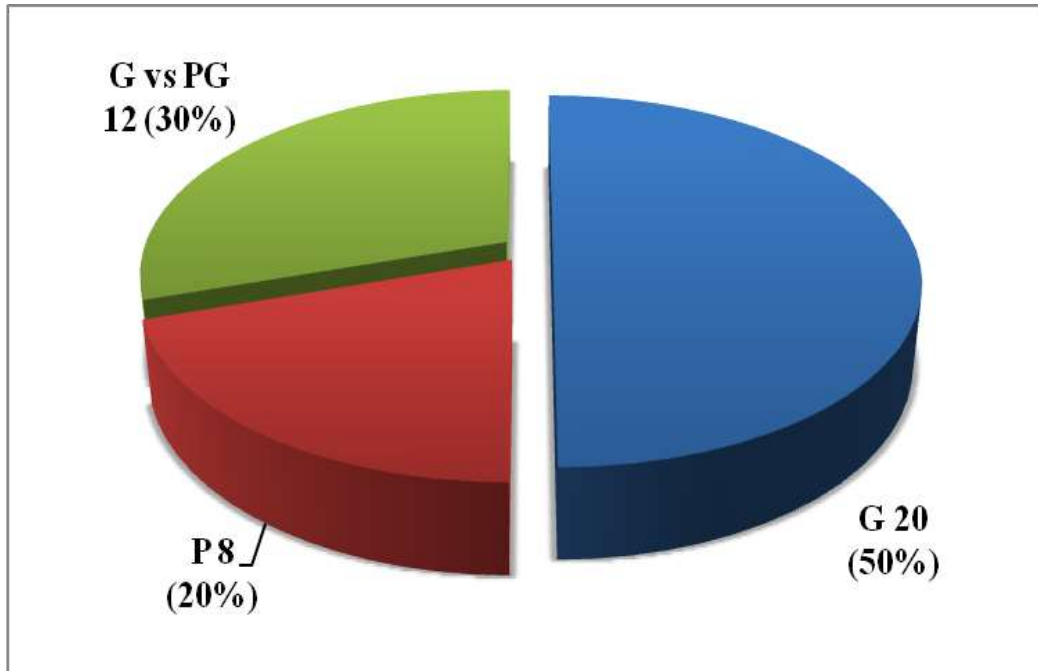


Figure 8: Histological diagnosis obtained. DGvsP: granulomatous vs pyogranulomatous dermatitis; SPGS: sterile pyogranuloma/granuloma syndrome; JC: juvenile cellulitis; NSP: nodular sterile panniculitis; RI: reactive histiocytosis.

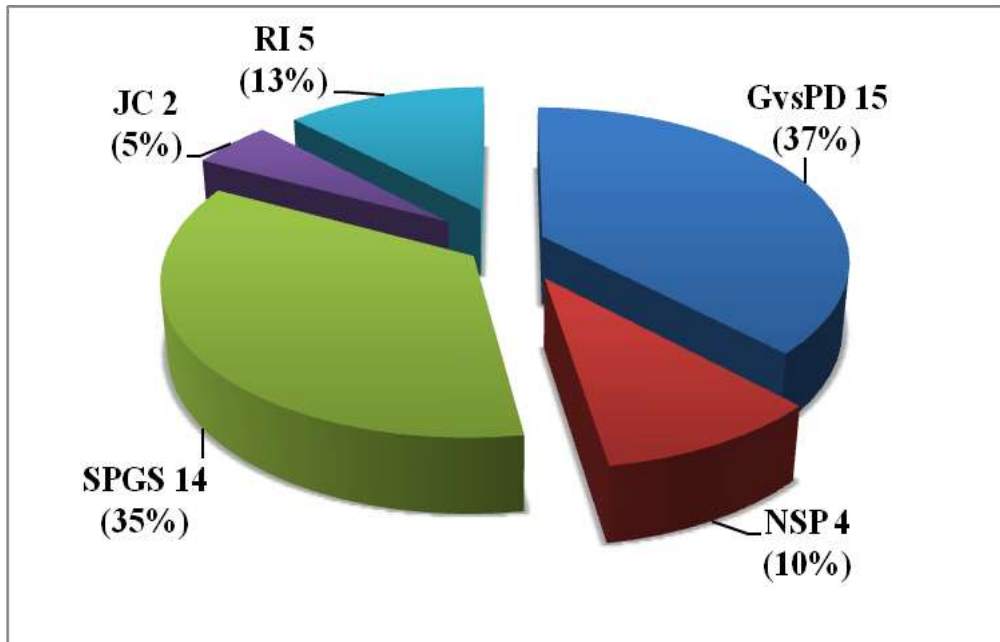


Figure 9: SPGS. A: Early inflammation is most prominent perivascular and strictly oriented along adnexa, with sausage-shaped or nodular pattern. B: The infiltrate is diffuse to the dermis in more severe forms (HE, 4X/HPF).

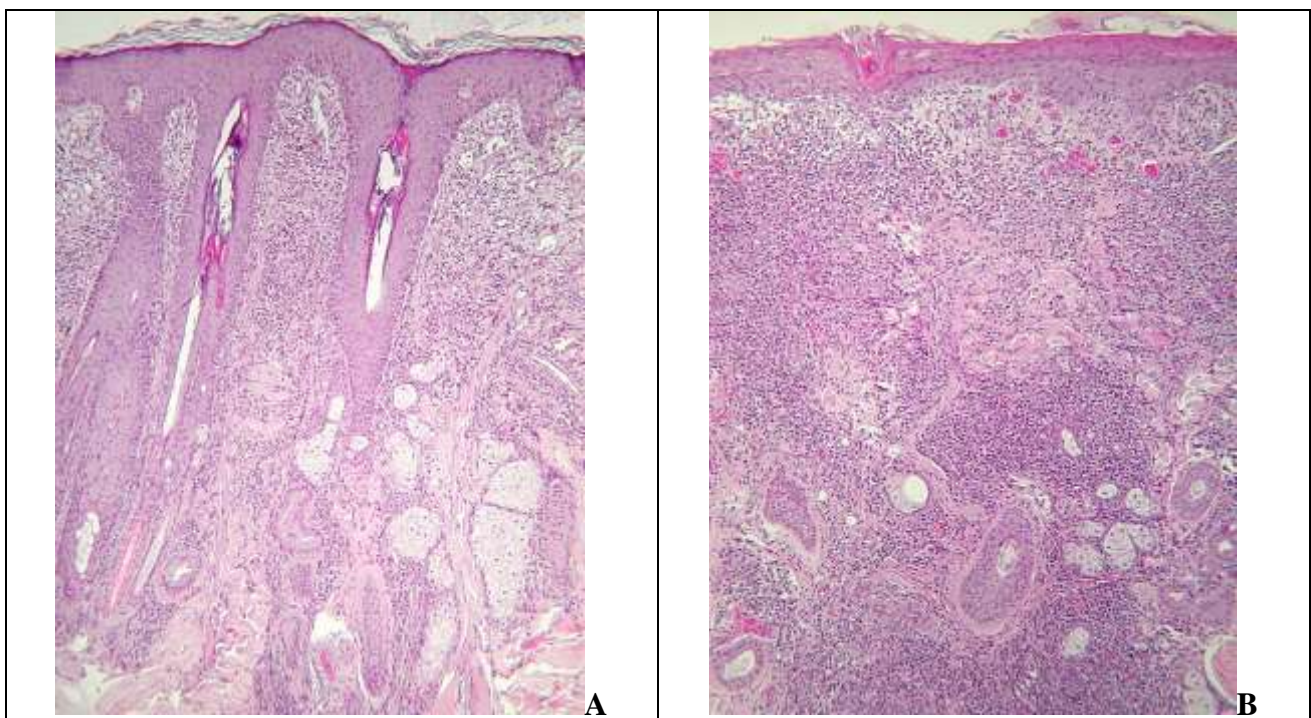


Figure 10: the immunohistochemistry was performed on all the selected samples. Only 1/40 was positive. The black arrows show the positivity (100/HPF)

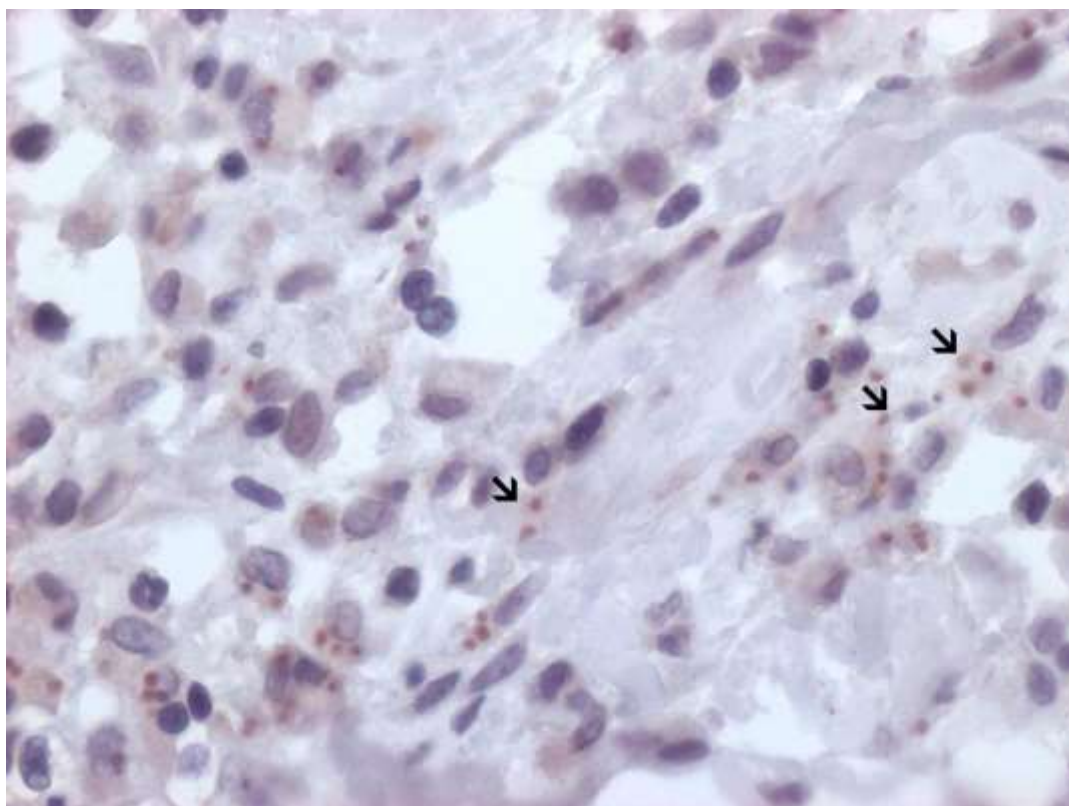


Figure 11. The Melt-Curve of RT-PCR for *Leishmania* spp. The stars show 3 positive samples

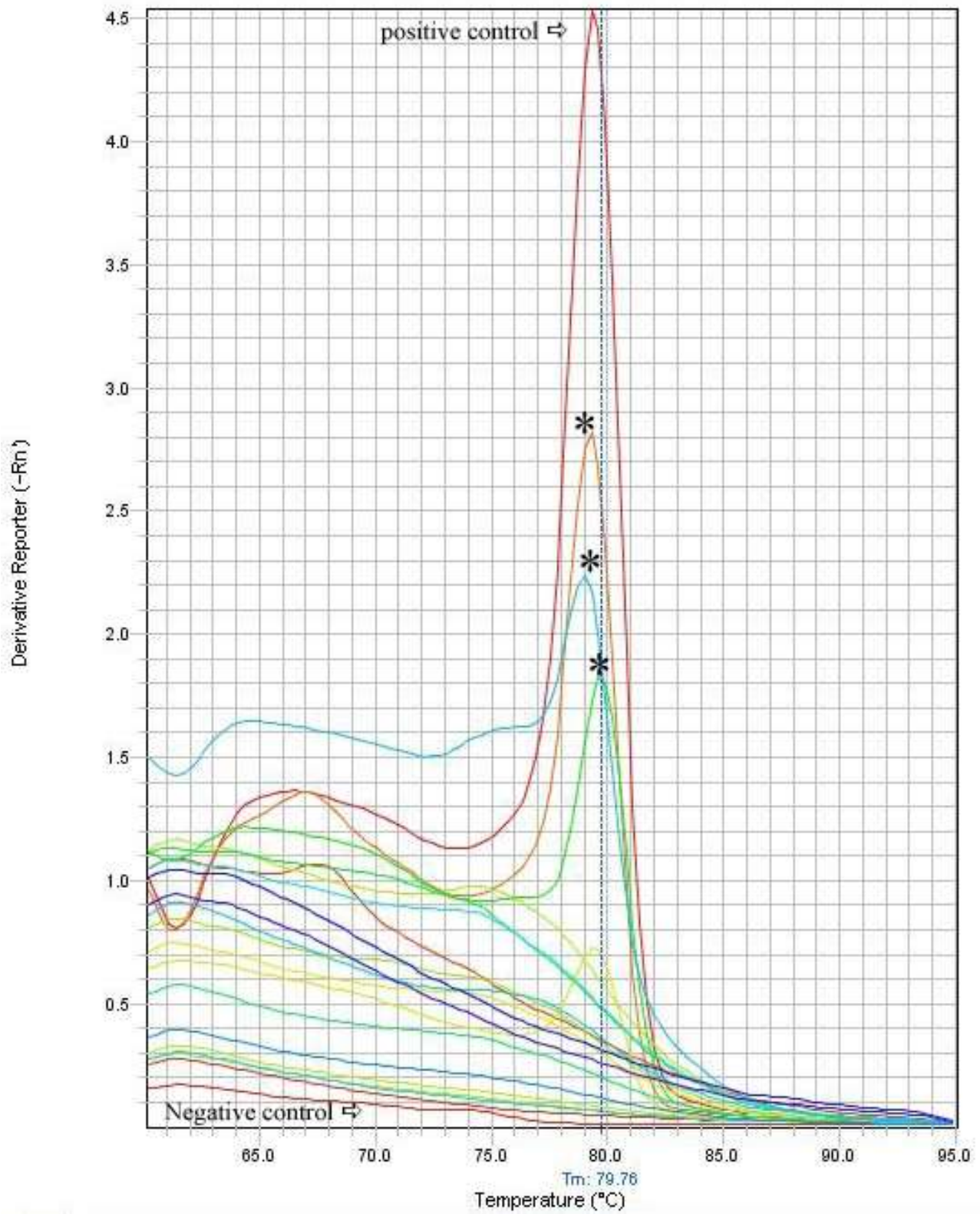
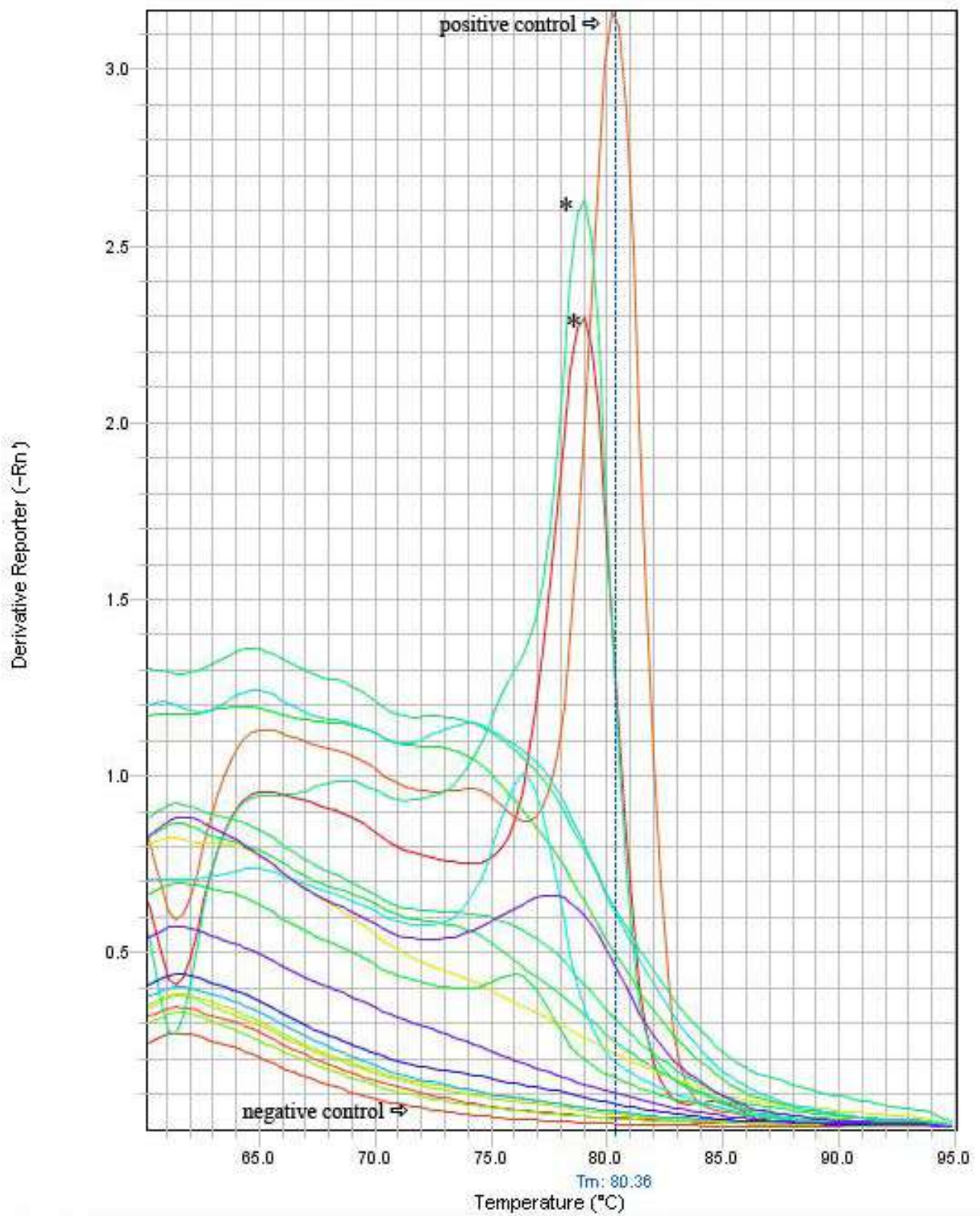


Figure 12. The Melt-Curve of RT-PCR for *Serratia marcescens*. The stars show 2 positive samples



Discussion

The diagnosis of SPGS, as for other “sterile” cutaneous granulomatous/pyogranulomatous skin lesions, is an elimination diagnosis of exclusion via the application of several ancillary techniques to biopsy samples (1–4,31). Among some of the techniques, the first to be utilized include examination of bacteriological and mycological cultures from fresh tissue samples (1–4,17). Unfortunately, in this research project, detection of microorganisms through culture examination was not possible in most cases, as this was mainly a retrospective study conducted on formalin fixed paraffin embedded samples. Even the new cases included came from paraffin embedded material and there was no possibility to perform bacteriological or/and mycological cultures prospectively. Special stains applied to histological sections of lesions consistent with SPGS were an aid in the detection or exclusion of the presence of microorganisms that cannot be evidenced by standard haematoxylin and eosin staining. Giemsa and Gram aid in the exclusion of bacteria, Periodic acid Schiff excludes the presence of fungi while Ziehl-Neelsen and Fite Faraco exclude the possible role of mycobacteria. The concomitant use of polarized light microscopy rules out the presence of foreign bodies (granulomatous reaction due to vegetable, organic, mineral and other materials). In our evaluation all these techniques were negative.

However, it is well known that any etiological agent present in small quantities or antigenic residues of the organism will be mostly undetected by special stains (1–4). In recent years, in an attempt to deal with these diagnostic limitations, methods applied in both veterinary and human medicine have included those based on the detection of microorganism DNA and antigenic proteins (11,12,32-36). The use of PCR, and recently RT-PCR, has allowed investigators to identify pathogens in lesions that were diagnosed previously as “sterile”. It has been reported that some apparently sterile granulomas are actually positive for *Leishmania* spp. when using immunohistochemical methods (immunoperoxidase) and PCR to detect this protozoan organism (6). In this study, we opted for a RT-PCR technique since it is considered more sensitive (37,38). A total of 4 out 40 samples

resulted RT-PCR positive for *Leishmania* spp. Interestingly, 3/4 of these samples had a negative PCR for *Leishmania* before their inclusion in the study; this might be related to the lower sensitivity of direct PCR compared to RT-PCR (11,12) and/or to inappropriate selection of paraffin embedded material or of the probes.

Noteworthy, of the 4 RT-PCR positive cases for *Leishmania* spp., only one case had a positive IHC. These results allow us to draw multiple conclusions. First, as it is a highly sensitive and specific technique, RT-PCR, according to some authors, is superior to immunohistochemistry (11,12). The greater sensitivity of RT-PCR is due to the ability to amplify DNA of microorganisms, thus enabling detection in more cases. It has been suggested that RT-PCR should be applied to all samples where immunohistochemistry gives uncertain or negative results. In the present study there was a difference in sensitivity between the techniques, and the methods were not comparable due to the limited number of cases. Second, IHC might be negative as a consequence of the inappropriate selection of paraffin embedded sections. For each skin biopsy, 15 sections had been obtained and processed. IHC was applied only on one 3 micron section thus, a negative result might be related to absence of the infective organism in that specific sample.

Another possibility is that, biopsies negative for microorganisms on microscopic examination may actually still have minimal amounts of the agent or of its residues. This is important with regard to *Leishmania* organisms in granulomatous skin lesions with histopathological features suggestive of SPGS. The samples were selected from institutions operating in areas endemic for Leishmaniosis. The presence of granulomatous lesions does not necessarily mean that these lesions are related to *Leishmania*, as the protozoan could reach the sites in already infected macrophages attracted during the inflammatory process (9,39). Based on our present data, it is impossible to state whether the histological lesion of SPGS is subsequent to the protozoan presence. This question may be answered by complete physical examination, haematological, biochemical and serological testing of dogs and evaluation of their response to therapy. Detection of high anti-*Leishmania* antibody titres

might be useful to differentiate between infected and ill dogs (40). Also it is important to note that, an animal in which *Leishmania* organisms are observed in tissue sections does not necessarily have or develop the disease (9,40). However, given the differences in treatment between SPGS and Leishmaniosis (2,7,8), RT-PCR should be applied to detect *Leishmania* DNA or RNA in all histological samples with the histopathologic diagnosis of SPGS in areas endemic for the disease. Because SPGS is a disease diagnosed by a process of elimination, and given the similarities with other granulomatous diseases associated with mycobacteria, the search for these microorganisms is mandatory. In both animals and humans, mycobacterial diseases may be difficult to diagnose. In humans, diseases that were diagnosed as sterile are now proved to be secondary to the presence of microorganisms or their residues include sarcoidosis (14) and several chronic enteropathies. Sarcoidosis shares some similarities with SPGS (41-42). In recent years, PCR and RT-PCR have allowed the identification of *Mycobacterium* spp. DNA in histological samples where both examination of bacterial and fungal cultures of fresh tissue and staining of paraffinized tissue elicited negative results (14,42,44). Even granulomas of canine leproid syndrome can be negative for the presence of mycobacteria. This disease is clinically similar to SPGS due to the localization of nodules on head and limbs and the lack of systemic signs of illness in affected dogs (45). The histopathological pattern is more similar to tuberculous granulomatous lesions, though in some cases the number of microorganisms is small, the lesion can be histologically compared to SPGS (8,45,46). In these cases, application of PCR for *Mycobacterium* spp. is essential for a correct diagnosis (45,46). In the present study, RT-PCR for mycobacteria was negative in all biopsies. The negative results could have been secondary to the scarcity of microbial DNA. This possibility could be addressed by using a larger number of histological paraffinized sections to extract and amplify DNA or RNA sequences. The second possible cause of negative results is the low specificity of the primers. However, the same technique was repeated and compared with samples whose positivity was already shown by special stains (positive controls) and the technique confirmed the presence of

mycobacteria. Our results are therefore unlikely to be negative as a consequence of laboratory errors (44).

All samples were collected in areas that are not endemic for mycobacterial diseases thus a possible mycobacterial infection should be considered as a rare to unlikely event, fact that supports further our results. The lack of clinical data about the animals did not allow us to make further comments on clinical presentation. Last, but not least, it would be interesting to apply this method to samples diagnosed as SPGS in areas considered endemic for mycobacterial disease (44-47) and where special stains are negative for infective agents.

Serratia marcescens is a gram negative bacillus classified as a member of *Enterobacteriaceae* (48).

It was considered initially as an innocuous, non pathogenic saprophytic water organism, but in the last decades it has become responsible of many nosocomial infections in man (48). The same problem has been reported in a veterinary hospital with outbreak of the infection and related environmental contaminations (49). In dogs, fatal infections related to *Serratia marcescens* have been reported, starting as nodular lesions and evolving into necrotizing fasciitis (50). All the collected samples were tested for *Serratia* spp. by RT-PCR and surprisingly two cases resulted positive. In these cases, special histochemical stains failed to reveal the infective agent. The greater sensitivity of RT-PCR, due to the ability to amplify DNA of microorganisms, might justify this result, when special stains resulted negative. This result was unexpected, unfortunately it was not possible to add new information such as a culture being both cases from old archival material (year 2010-2011). The histological pattern was not helpful to distinguish among the bacterial etiology and cases of SPGS. Thus, in some cases *Serratia marcescens* could have been missed and lesions misdiagnosed as SPGS. Further investigation of the role of *Serratia* spp. In canine nodular skin disease is warranted due to the lack of any information and knowledge of the lesional profile of *Serratia* spp. in infected skin in dogs. However, the two positive strains at melting curve analysis showed an anticipation of peak with T_m at 79°C respect to the reference strain with T_m 80.36°C . It

would be interesting to proceed to the sequencing of the two strains amplified to confirm or refute this result.

Nocardia spp. are microorganisms ubiquitous, soil saprophytes, found in organic material, water and plants (51). Pathogenicity of these species is influenced by bacterial intracellular localization, induction of pyogranulomatous inflammatory response, host susceptibility, via of transmission and co-infection with immunosuppressive diseases. These facultative intracellular pathogens are able to inhibit phagosome-lizosome fusion, and resist to acid, oxidative and enzyme mechanisms into neutrophils and macrophages, due to presence of mycolic acids in bacterial cell wall, resulting in severe tissue inflammatory processes (51). Furthermore, Nocardiosis appears in several clinical forms. Skin lesions may be represented by pyoderma and/or pyogranulomatous dermatitis (52). For these reasons, Nocardiosis should be always present in the list of differential diagnoses of cutaneous nodular lesions. In this study, this infective agent was not detected both by special stains and RT-PCR. As for *Serratia marcescens*, *Nocardia spp.* does not seem to be a possible agent of SPGS. Finally BCG and special stains failed to reveal any pathogen within the exanimate samples supporting the RT-PCR results.

In conclusion, *Leishmania spp.* and *Serratia marcescens* were the only infective agent identified in a minority of skin lesions with a diagnosis of cutaneous sterile nodular lesions. Further studies regarding other bacterial or protozoal organisms might lead to different conclusions.

References

1. Torres SMF. Sterile nodular dermatitis in dogs. *Veterinary Clinics of North America* 1999; 29: 1311–23.
2. Miller WJ, Griffin GE, Campbell KL. Miscellaneous skin diseases. In: Muller and Kirk's *Small Animal Dermatology*. 7th ed. Elsevier Inc., 2013: 695-723.
3. Gross TL, Ihrke PJ, Walder EJ. Non-infectious nodular and diffuse granulomatous and pyogranulomatous diseases of the dermis. In: *Veterinary Dermatopathology: a Macroscopic and Microscopic Evaluation of Canine and Feline Skin Diseases*. St Louis, MO: Mosby Year Book, 1992: 194–8.
4. Yager JA, Wilcock BP. Nodular and/or diffuse pyogranulomatous dermatitis. In: *Color Atlas and Text of Surgical Pathology of the Dog and Cat*. St Louis, MO: Mosby Year Book, 1994: 141–3.
5. DeManuelle TC, Stannard AA. Difficult dermatologic diagnosis. Sterile nodular panniculitis. *Journal of the American Veterinary Medical Association* 1998; 213: 356–7.
6. Cornegliani L, Fondevila D, Vercelli A, Fondati A. PCR technique detection of *Leishmania* spp but not *Mycobacterium* spp. in canine cutaneous “sterile” pyogranuloma/granuloma syndrome. *Veterinary Dermatology*, 2005; 16: 233-238.
7. Miller WJ, Griffin GE, Campbell KL. Viral, rickettsial, and protozoal skin diseases. In: Muller and Kirk's *Small Animal Dermatology*. 7th ed. Elsevier Inc., 2013: 343-362.
8. Gross TL, Ihrke PJ, Walder EJ. Infectious nodular and diffuse granulomatous and pyogranulomatous diseases of the dermis. In: *Veterinary Dermatopathology: a Macroscopic and Microscopic Evaluation of Canine and Feline Skin Diseases*. St Louis, MO: Mosby Year Book, 1992: 169–71, 186–8.

9. Font A, Roura X, Fondevila D et al. Canine mucosal leishmaniasis. *Journal of the American Animal Hospital Association* 1996; 32: 131–7.
10. Ferrer L, Rabanal RM, Domingo M et al. Identification of *Leishmania donovani* amastigotes in canine tissues by immunoperoxidase staining. *Research in Veterinary Science* 1988; 44: 194–6.
11. Roura X, Fondevila D, Sánchez A et al. Detection of *Leishmania* infection in paraffin-embedded skin biopsies of dogs using polymerase chain reaction. *Journal of Veterinary Diagnostic Investigation* 1999; 11: 385–7.
12. Roura X, Sanchez A, Ferrer L. Diagnosis of canine leishmaniasis by polymerase chain reaction technique. *Veterinary Record* 1999; 144: 262–4.
13. Drake WP, Pei Z, Pride DT et al. Molecular analysis of sarcoidosis tissues for *Mycobacterium* species DNA. *Emerging Infectious Diseases* 2002; 8: 1334–41.
14. Hsiao PF, Tzen CY, Chen HC et al. Polymerase chain reaction based detection of *Mycobacterium tuberculosis* tissues showing granulomatous inflammation without demonstrable acid-fast bacilli. *International Journal of Dermatology* 2003; 42: 281–6.
15. Carlotti DN, Mathet JL, Bensignor E: Aspects cliniques et histopathologiques des pseudo-neoplasmes cutanes chez le chien, le chat, le cheval et les ruminants i granulomes et pyogranulomes. *Le Point Veterinaire* 2000; 31: 45-55.
16. Santoro D, Prisco M, Ciaramella P: Cutaneous sterile granulomas/pyogranulomas, leishmaniasis and mycobacterial infections. *Journal of Small Animal Practice* 2008; 49: 552-561.
17. Panich R, Scott DW, Miller WH. Canine cutaneous sterile pyogranuloma/granuloma syndrome: a retrospective analysis of 29 cases (1976–1988). *Journal of the American Animal Hospital Association* 1999; 7: 519–28.
18. Diaz SF, Gilbert S: oral lesions in a case of sterile granuloma and pyogranuloma syndrome. *Veterinary Dermatology* 2007; 18: 179.

19. Barret SJ, Sheafor SE, Hillier A, Chew DJ. Challenging cases in internal medicine: what's your diagnosis? *Veterinary Medicine* 1998; 93: 35-44.
20. Jeffer JG, Duclos DD, Goldschmidt MH. A dermatosis resembling juvenile cellulitis in a adult dog. *Journal of American Animal Health Association* 1995; 31: 204-208.
21. Scott DW, Noxon JO. Idiopathic sarcoidal granulomatous skin disease. *Canadian Practice* 1990; 15: 11-18.
22. Baines SJ, McInnes EF, McConnell I. E- cadherin expression in canine cutaneous histiocytomas. *Veterinary Records* 2008; 8: 509-513.
23. Bonenberger TE, Ihrke PJ, Naydan DK, Affolter VK. Rapid identification of tissue microorganisms in skin biopsy specimens from domestic animals using polyclonal BCG antibody. *Veterinary Dermatology* 2001; 12(1): 41-7.
24. Rodgers MR, Popper SJ, Wirth DF. Amplification of kinetoplast DNA as a tool in the detection and diagnosis of *Leishmania*. *Experimental Parasitology* 1990; 71(3): 267-75.
25. Park H, Jang H, Kim C. et al. Detection and identification of *Mycobacteria* by amplification of the internal transcribed spacer regions with genus and species-specific PCR primers. *Journal of Clinical Microbiology*. 2000, 38: 4080–4085.
26. Iwaya A, Nakagawa s, Iwakura N et al., Rapid and quantitative detection of blood *Serratia marcescens* by a real-time PCR assay: Its clinical application and evaluation in a mouse infection model *FEMS Microbiology Letters* 2005; 248 (2): 163–170.
27. Alfaresi M, Elkosh A. Rapid identification of clinically relevant *Nocardia* species using real-time PCR with SYBR Green and melting-curve analysis *J Med Microbiol* December 2006;. 55 (12) 1711-1715.
28. Wittwer CT, Herrmann MG, Moss AA, Rasmussen RP. Continuous fluorescence monitoring of rapid cycle DNA amplification. *Biotechniques*, 1997; 22: 130–138.

29. Higuchi R, Fockler C, Dollinger G and Watson R. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. *Bio-Technology* 1993; 11, 1026–1030.
30. Ririe KM, Rasmussen RP, Wittwer CT. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Anal Biochem*, 1997; (15) 245, 154–160.
31. Ihrke PJ, White SD. Difficult dermatologic diagnosis. *Journal of the American Veterinary Medical Association* 1998; 213: 356–7.
32. Aranaz A, Liébana E, Pickering X et al. Use of polymerase chain reaction in the diagnosis of tuberculosis in cats and dogs. *Veterinary Record* 1996; 138: 276–80.
33. Brecelj M, Pikelj F, Gubensek F et al. Polymerase chain reaction as a diagnostic tool for detecting *Leishmania*. *Infection* 2000; 28: 111–13.
34. Muller N, Zimmermann V, Foster U et al. PCR-based detection of canine *Leishmania* infections in formalin-fixed and paraffin-embedded skin biopsies: elaboration of a protocol for quality assessment of the diagnostic amplification reaction. *Veterinary Parasitology* 2003; 114: 223–39.
35. Osaki M, Adachi H, Gomyo Y et al. Detection of mycobacterial DNA in formalin-fixed, paraffin-embedded tissue specimens by duplex polymerase chain reaction: application to histopathologic diagnosis. *Modern Pathology* 1997; 10: 78–83.
36. Ikonopoulou JA, Gorgoulis VG, Zacharatos PV et al. Multiplex polymerase chain reaction for the detection of mycobacterial DNA in cases of tuberculosis and sarcoidosis. *Modern Pathology* 1999; 12: 854–62.
37. Paiva-Cavalcanti M, Regis-da-Silva CG, Gomes YM. Comparison of real-time PCR and conventional PCR for detection of *Leishmania (Leishmania) infantum* infection: a mini-review. *The Journal of Venomous Animals and Toxins including Tropical Diseases* 2010, 16 (4): 537-542.
38. Do Nascimento Ramos RA, Do Nascimento Ramos CA, Gomes Jusi MM et al. Polymerase chain reaction and real-time PCR for diagnosing of *Leishmania infantum chagasi* in dogs. *Rev. Bras. Parasitol. Vet.*, Jaboticabal, 2012, 21 (3): 192-195.

39. Fondevila D, Vilafranca M, Ferrer L. Epidermal immunocompetente in canine leishmaniasis. *Veterinary Immunology and Immunopathology* 1997; 56: 319–27.
40. Ferrer L, Solano-Gallego L, Arboix M et al. Evaluation of the specific immune response in dogs infected by *Leishmania infantum*. In: Thoday KL, Foil CS, Bond R eds. *Advances in Veterinary Dermatology*, Vol. 4. Oxford, UK: Blackwell Publishing Co., 2002: 92–9.
41. Newman LS, Rose CS, Maier LA. Sarcoidosis. *New England Journal of Medicine* 1997; 336: 1224–34.
42. Mendel J, Weinberg SE. Clinical insights and basic science correlates in sarcoidosis. *American Journal of Medical Science* 2001; 321: 99–107.
43. Shapiro PE. Non infectious granulomas. In: Elder D, Elenitsas R, Jaworsky C et al. eds. *Lever's Histopathology of the Skin*, 8th edn. Philadelphia: Lippincott-Raven Publishers, 1997: 315–40.
44. Salian NV, Rish JA, Eisenach KD et al. Polymerase chain reaction to detect *Mycobacterium tuberculosis* in histologic specimens. *American Journal of Respiratory and Critical Care Medicine* 1998; 158: 1150–5.
45. Foley JE, Borjesson D, Gross TL et al. Clinical, microscopic, and molecular aspects of canine leproid granuloma in the United States. *Veterinary Pathology* 2002; 39: 234–9.
46. Hughes MS, James G, Ball N et al. Identification by 16S rRNA gene analyses of a potential novel mycobacterium species as an etiological agent of canine leproid granuloma syndrome. *Journal of Clinical Microbiology* 2000; 38: 953–9.
47. Lemarie SL. Mycobacterial dermatitis. *Veterinary Clinics of North America* 1999; 29: 1291–301.
48. Hejazi A, Falkiner FR. *Serratia marcescens*. *Journal of Medical Microbiology* 1997; 46: 903–912.

49. Fox GJ, Beaucage CM, Folta CE et al. Nosocomial transmission of *Serratia marcescens* in a veterinary hospital due to contamination of benzalkonium chloride. *Journal of Clinical Microbiology* 1981,14 (2): 157-160.
50. Plavec T, Zdovc I, Juntos P et al. Necrotizing fasciitis caused by *Serratia marcescens* after tooth extraction in a Doberman pinscher: a case report. *Veterinarni Medicina* 2008; 53 (11): 629-635.
51. Ribeiro MG, Salerno T, De Mattos-Guaraldi AL et al. Nocardiosis: an overview and additional report of 28 cases in cattle and dogs. *Rev. Inst. Med. trop. S. Paulo* 2008; 50 (3): 177-185.
52. Golynski M, Szczepanik M, Pomorska D et al. Cutaneous nocardiosis in a dog – clinical case presentation. *Bull Vet Inst Pulawy* 2006; 50: 47-50.

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