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Detection of Antinuclear Antibodies in Canine Serum by Immunoperoxidase in MDCK and Indirect Immunofluorescence in HEp2 Cells

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Abstract

Antinuclear antibodies are found in animals suffering from Systemic Lupus Erythematosus (SLE) and some other diseases, their presence in the blood is determined by antinuclear antibody (ANA) test using indirect immunofluorescence (IF) with HEp2 cells as a substrate. In this work, an immunoperoxidase (IP) assay was developed to evaluate the ANAs in canine sera, using canine kidney cell lines (MDCK) and compared with a commercial immunofluorescence test on Hep2 cells for this system, a fluoresceinated anti-canine Ig antibody was standardized. The study was performed on 50 sera from dogs submitted to the laboratory with different clinical diagnoses of autoimmune-associated diseases. The procedures on both cells were unified to perform comparisons of the reactions, direct sera or at different dilutions were added to a monolayer of permeabilized MDCK cells, followed by a peroxidized anti-canine IgG conjugate, and a substrate for the IP reaction. The same sera were tested on the commercial IF assay on Hep2 cell system. In 22/50 cases, the presence of LE cells in peripheral blood was determined. A high correlation was found in the detection of antinuclear antibodies between both cell lines and techniques, however there were differences in the reaction patterns in the nucleus and cytoplasm between cell lines. The diffuse nuclear pattern observed in MDCK cells was more related to the presence of high percentages of LE cells in peripheral blood. The differences found in the results were possibly associated with the presence of homologous antigens between the MDCK cells and the dog. In addition, the methodology and standardization for the use and interpretation of a reference serum was developed to unify the interpretation criteria in the laboratory.

Keywords

Antinuclear Antibodies, Immunoperoxidase in Marbin Darbin Canine Kidney (MDCK), SLE Associate Disease

1. Introduction

Circulating antinuclear antibodies (ANA) and some cytoplasmic autoantibodies are commonly present in SLE and other systemic rheumatic diseases, in humans and dogs. The indirect immunofluorescence (IF)-ANA test is the standard method for detecting ANA. The presence of elevated ANA titers is a sensitive marker of SLE in both humans and dogs [1]. ANAs are antibodies with specificity for nucleic acids and nucleoproteins. Although these antibodies are not present in healthy individuals, low levels are sometimes found in elderly patients or transiently in patients after trauma. Some infections can induce the development of a positive ANA test, thus elevated serum levels (titers >1:80) are associated with autoimmune diseases.

Substrates employed for ANAs detection have included mouse and rat liver sections, and monolayers of HeLa, Vero, human hepatoma, or HEp-2 cells. The sensitivity of the assay varies between laboratories and the substrate employed, and the interpretation of IF results is highly subjective and depends on the source of the tissue/cellular substrate [2] The Hep-2 cells were found to be superior to rat liver cryosections due to the low reactivity with normal sera, and also provided easier discernment [3]. The anti-nuclear antibodies react to nuclear antigens other than histones and there are antigens whose concentration in tissue cells is considerably lower in contrast to Hep2 cells. Another characteristic of these cells is that they have more than 46 chromosomes, more than two nucleoli and, being very metabolically active, have many mitochondria and their nucleus is bigger than any normal epithelial cell, so it is considered the best screening test for ANA in humans [2] [4]. However, there is no international standard by which the test should be performed, and the individual laboratory should advise on the significance of the titer, as it may depend on the methodology employed [5].

In veterinary medicine, the IF-ANA test with HEp2 cells is used in the diagnosis of canine SLE and canine SLE-related disorders. In canine samples, distinct immunofluorescence labeling patterns can be identified when HEp2 cells are used as substrate. The most frequent are the homogenous pattern, with chromosomal staining of mitotic cells and the speckled pattern, with non-chromosomal staining. Others less frequent patterns are the rim, and nucleolar labeling. In these cells, a homogenous pattern is mainly associated with reactivity against dsDNA and DNA-associated proteins, while a speckled pattern is mainly associated with specific ANA against anti-ribonucleoproteins (tRNP), anti-Smith (Sm), Sjögren's syndrome related antigens (SS-A, SS-B); also called

extractable nuclear antigens (ENAs) [1].

In dogs with SLE, ANAs typically recognize histones (61% - 74% of cases) or soluble nuclear antigen, whereas human ANAs favor double-stranded DNA as antigen. However, the presence of anti-double-stranded DNA in dogs is controversial. In canine SLE, dogs may have antibodies to Sm proteins and tRNP, but previous research demonstrated that histones are the most common nuclear substrate for ANA in dogs with SLE., furthermore, 43 kDa nuclear proteins, is the common nuclear epitope for autoantibodies in dogs, and this is a prominent canine autoantigen [2].

The presence of antihistone antibodies is highly correlated with a positive diagnosis of dog SLE. In one study, about 70% of dogs meeting American College of Rheumatology (ACR) criteria [6], involvement of at least four body systems, had antihistone antibodies, compared to 6.7% of control dogs. The pattern of recognition, however, differed from that found in humans. Using immunoblots, 54% of sera from dogs with SLE recognized H4 and H3, 8% recognized H1, 22% H2A, and 20% H2B. In another study, no antibodies to H2B were detected in any of 43 sera from dogs with SLE. In contrast, H1 and H2B are more prominent autoantigens in humans. Early studies had demonstrated that anti-T1 (hnRNP G) gives a relatively fine speckled pattern, anti-Sm and anti-RNP give a coarser speckled, or reticle nodular pattern, and the antihistone antibody gives a generally homogeneous pattern. The nucleosome consists of the repeat length DNA, a histone octamer and one H1 molecule, the lysine rich H1 histone family in mammals includes eleven different subtypes, and thus it is the most divergent class of histone proteins. Interestingly, the distribution of these eleven histone genes is highly conserved between the human, mouse, and rat genomes and this allows the distinction between two types of histone gene arrangements, namely clustered versus solitary histone genes, which have a three-domain structure.

The short N-terminal domain is about 45 amino acids long and is enriched in basic amino acids in its second half adjacent to the central domain. This central portion of the protein includes about 75 amino acids, is highly conserved among all H1 subtypes and has a globular conformation. The C-terminal domain has a length of about 100 amino acids and is highly enriched in lysine, serine and proline, this portion of the protein is unstructured in aqueous [2].

When analyzing the correlation between antinuclear reactions of human sera in HEp2 and the reaction with recombinant antigens in ELISA plates, differences are observed that confirms that the antibodies recognize conformational epitopes [4], reinforcing the possibility of antigenic differences between humans and canines patients.

ANA detection by IF in Hep2 cell lines is considered the initial test supporting the diagnosis of autoimmune diseases due to its high sensitivity. However, given its relatively low specificity, it is necessary to use more sensitive and specific techniques such as radioimmunoassay (RIA), ELISA, electroimmunotransference (EIT), Western blot or to change to a more appropriate substrate in terms of antigenicity to the species under study to increase the sensitivity and specific-

ity of ANAs for the diagnosis of autoimmune diseases [4] [7].

2. Objectives

The aim of this study was to compare the reactivity and specificity of antinuclear antibodies in HEp2 and MDCK cells by immunofluorescence and immunoperoxidase and to correlate the patterns in the nucleus with the LE cell test. In addition, it was intended to obtain a standard positive canine serum for use in MDCK cell testing.

3. Methods

Fifty sera from canines submitted to the laboratory with a clinical diagnosis of an autoimmune disease were evaluated. Antinuclear antibodies were evaluated with a commercial antinuclear antibody kit for human use, based on HEp2 cells purchased in Mexico. The anti-human antibody was replaced with a Sigma™ fluresceinated anti IgG-canine antibody at a dilution of 1:800 and the manufacturer's instructions were followed. The 50 tested sera were added to the slides, directly or at 1:40, 1:80 and 1:160 dilutions, incubated at room temperature for 1 hour. After washing, fluorescent anti-canine IgG was added. The slides were incubated and washed to be mounted with a coverslip. They were evaluated on an Olympus™ microscope associated with a 100 W epifluorescence device. Photographs were taken and analyzed.

An assay was developed using MDCK cell cultures grown to 80% confluency on coverslips in MEM medium supplemented with 7% fetal bovine serum (FBS). Cells were fixed with methanol and permeabilized with acetone for 15 minutes. The 50 sera were assayed directly or at 1:40, 1:80 and 1:160 dilutions, added to coverslips with cells, incubated at room temperature for 1 hour and washed with PBS-Tween. 100uL of peroxidized anti-canine IgG antibody (Sigma™) was added at a dilution of 1:200 for 1 hour at 37˚C, twice washed and the multiplex immunohistochemistry detection system (Biocare Medical™) was added for 20 min. to finally wash cells, IntelliPath™ auto Stainer was used for all procedures described. Finally, the stained cells were observed on an Olympus™ microscope. To be considered a positive sample, at least 30% of the cells had to show the reaction pattern. The reaction patterns on the cells were classified according to [8]. To rule out natural autoantibodies only sera with titers greater than or equal to 1:80 were considered positive [9].

Only in 22 of the cases was it possible simultaneously obtain a heparinized blood sample to perform the LE cell technique. The protocol described by [10] was used as follows: the blood sample containing glass beads, were shaken, and was incubated at 37˚C for 2 h, capillaries were filled and centrifuged at 13,000 rpm. Several buffy coat smears were made, fixed with methanol, and stained with Wright's for microscopic observation. 1000 leukocyte cells were counted, and the percentage of LE cells was scored.

Two sera that resulted in positive titers of 1:160 in the MDCK cells were tested

10 times, and evaluated by 3 different pathologists (GVA, ACU, AEP). The positive reaction, pattern, and grade (mild (+), moderate (++) and severe (+++)) were established in accordance with [11]. The Fleiss Kappa test was utilized to determine the interrater reliability using IBM SPSS Statistics program (V.25).

4. Results

The results were classified by the reactions observed in the nucleus of both techniques employed according to [1], as: negative (NN), perinuclear (PN), diffuse (ND), coarse granular (speckled pattern) (GG), fine granular speckled pattern (GF), with non-chromosomal staining (NC) (**Figures 1-3**) Results are summarized in **Table 1(a)** and **Table 1(b)**. Was considered positive for ANA any nuclear reaction pattern (Perinuclear, diffuse, coarse granular, speckled pattern, fine granular speckled pattern, or with non-chromosomal staining) if more of 30% of the cells had the nuclear pattern at a titer equal to or greater than 1:80.

In HEp2 cells, thick speckled patterns (GG) were found in 50% of the sera (anti-SM and anti-RPN) and in 85% with a perinuclear pattern (PN) without finding the homogeneous pattern (Anti histone). In contrast, MDCK cells showed 60% homogeneous pattern, 30% coarse granular pattern, and 55% perinuclear pattern. The deposit of antibodies against cytoplasmic components was very similar in both cells with 85% in HEp2 and 95% in MDCK. The parameters found (**Table 1(b)**) were sensitivity = 66.7%, specificity = 85.7%, positive predictive value = 66.7% and negative predictive value = 85.7%.

In both cell lines, a combination of reaction patterns was found in the nucleus.

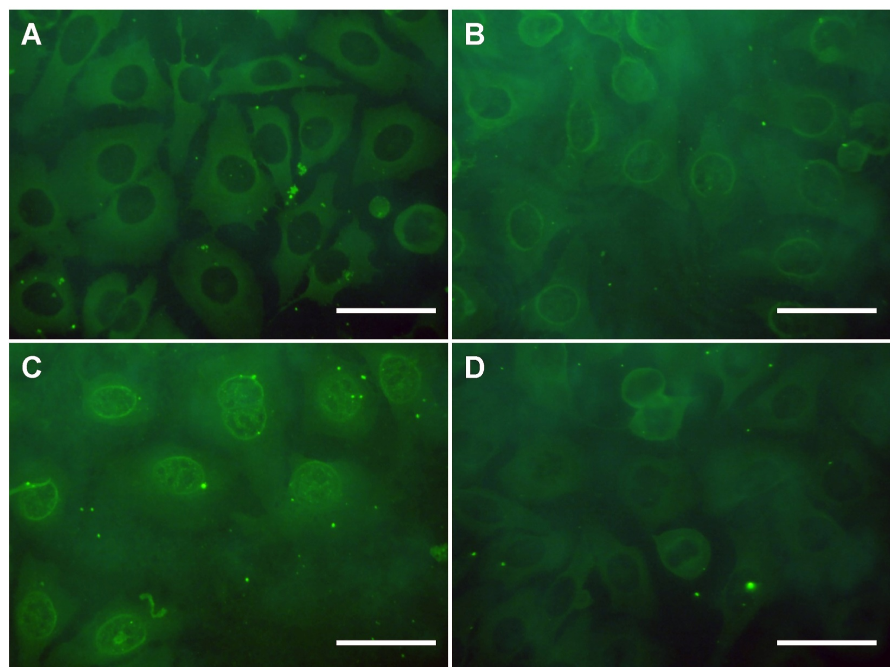

Figure 1. HEp2 cells, IIF, 400×, (a). Nuclear Negative (NN); (b). Perinuclear (PN); (c). Perinuclear (PN) and Coarse granular (speckled pattern) (GG); (d). Speckled pattern, with non-chromosomal staining (NC). Bar = 50 μm.

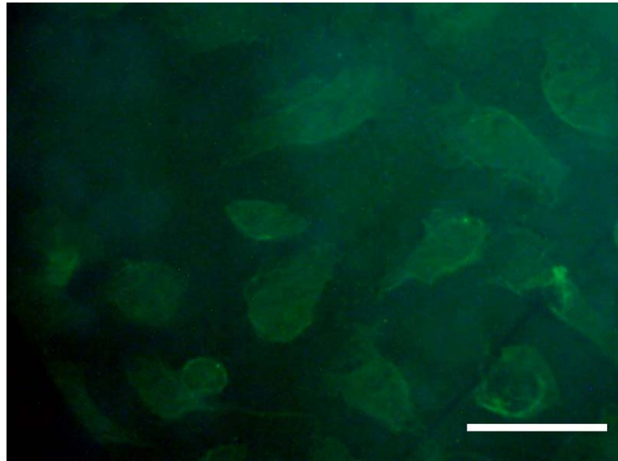

Figure 2. HEp2 cells IF, 400× showing a diffuse pattern (ND). Bar = 50 μm.

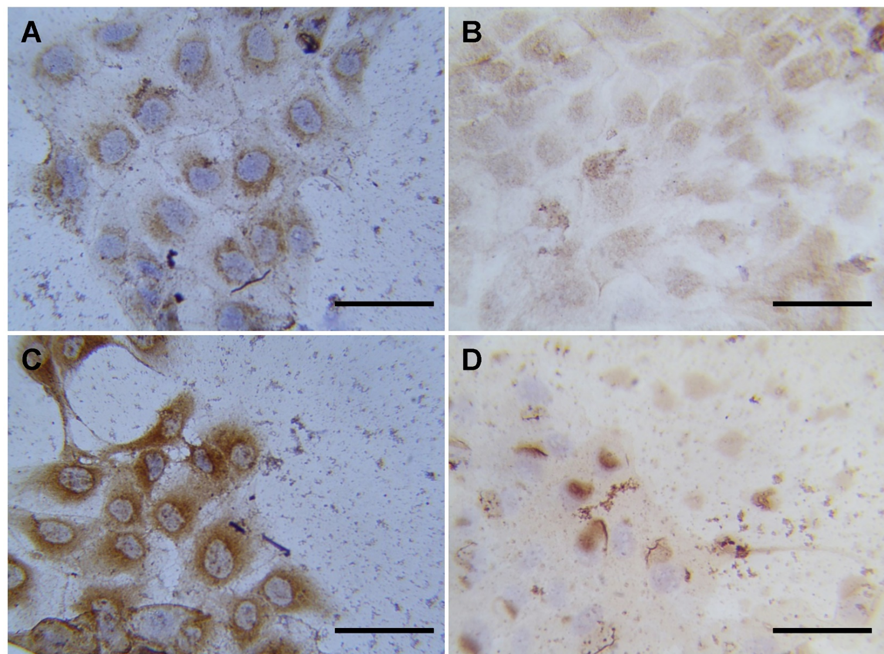

Figure 3. MDCK cells, immunoperoxidase, 400×, (a). Nuclear Negative (NN); (b). Fine granular (GF); (c), Perinuclear (PN) and coarse granular (speckled pattern) (GG); (d). Diffuse pattern (ND). Bar = 50 μm.

The percentage of LE cells was calculated and associated with the ANA results in Hep2 and MDCK cells for each case from which it was possible to perform both techniques (Table 2). Considering more than 5% of LE cells, in 18.2% (4/22) of cases, the sera presented a diffuse nuclear pattern (ND), or coarse granular (speckled pattern) (GG) on MDCK cells and a thick granular pattern (speckled pattern) on HEp2 cells.

To analyze the relationship between the result of the observation of LE cells. with the patterns found in the nucleus of HEp2 and MDCK cells, % LE cells were categorized as negative, low (1% - 5%), moderate (6% - 10%) and high (>11%)

Table 1. (a) Correlation of results between MDCK and Hep2 cells. (b) Sensitivity and specificity by SPS™.

(a)

	Hep2 (+)	Hep2 (−)	
MDCK (+)	10	5	15
MDCK (−)	5	30	35

Antinuclear antibodies (ANA) Positive (+): any nuclear reaction pattern; Perinuclear, diffuse, coarse granular speckled pattern, fine granular speckled pattern, with non-chromosomal staining in at least 30% of the cells at a titer equal to or greater than 1:80.

(b)

Cross tab: MDCK*HEP-2					
			HEP-2		Total
			negative	positive	
MDCK	negative	Count	30	5	35
		% in HEP-2	85.7%	33.3%	70.0%
	positive	Count	5	10	15
		% in HEP-2	14.3%	66.7%	30.0%
Total		Count	35	15	50
		% in HEP-2	100.0%	100.0%	100.0%

A significance level of $p < 0.05$ was used on **Table 1**.

Table 2. Percentage of LE-positive cells related to the ANA in HEp2 and MDCK cells.

Case id	nuclear pattern in Hep2 cells	nuclear pattern in MDCK cells	Percentage of positive LE cells
1	Perinuclear, coarse granular (speckled pattern)	Diffuse (ND)	12%
2	Perinuclear, coarse granular (speckled pattern)	Diffuse (ND)	8%
3	Perinuclear, coarse granular (speckled pattern)	Diffuse (ND), Coarse granular (speckled pattern) (GG)	22%
4	Perinuclear	Coarse granular (speckled pattern) (GG)	9%
5	Negative	Diffuse (ND), perinuclear	4%
6	Negative	Negative	2%
7	Negative	Negative	5%
Several (n = 15)	Negative	Negative	Negative

Total n = 22, Negative = no reaction observed in the nucleus.

Table 3. Results on MDCK in two positive sera.

Serum	Pattern	Observer 1	Observer 2	Observer 3
A	Coarse granular (speckled pattern) (GG),	10/10 (++)	9/10 (++) 1/10 (+++)	10/10 (++)
	Perinuclear (PN)	10/10 (++)	10/10 (++)	10/10 (++)
	Fibrillar citoplasm	10/10 (+++)	10/10 (+++)	10/10 (+++)
B	Coarse granular (speckled pattern) (GG),	9/10 (+) 1/10 (++)	10/10 (+)	10/10 (+)
	Perinuclear (PN)	10/10 (++)	10/10 (++)	10/10 (++)
	Fibrillar citoplasm	9/10 (+) 1/10 (++)	10/10 (++)	10/10 (++)

Sera A and B: dilution 1:160, degrees in observation: mild (+), moderate (++), severe (+), 10 replicates.

and the analysis was performed using the Chi-square test (SPSS Statistic™), this analysis indicated that in both cell lines the correlation was significant between a negative result to LE cells and no nuclear reaction ($p < 0.001$) Low, moderate or high results had no significant correlation with nuclear patterns in both cells ($p > 0.05$).

According to the reactions observed in the cytoplasm of both cell lines, the cytoplasmic patterns found in the present work were fibrillar (CF), speckled (CS), reticular/mitochondrial (CR), polar/Golgi (CP) and rods and rings (RR).

5. Discussion

The definition of the different ANA patterns determined by immunofluorescence in HEp2 cells must include the analysis of cells in interphase and active division, since there are antigens that are only expressed in certain phases of the cell cycle [4]. Furthermore, the subjectivity in the reading of ANA IF can hinder the expected standardization in laboratories. In this context, the differences between substrates, the rigor of the washing steps, the avidity and specificity of the conjugate, as well as the type of microscope, significantly increase intra- and inter-laboratory variability [12], all these factors of misinterpretation were tried to minimize during the experiments.

According to [13] [14], after trypsinization and passage of the MDCK cells, incubation with 3% FBS for three hours allows synchronization in 60% - 70% of the growth of the cells, and subsequent incubation for 48 hours with 7% FBS allowed these cells to be in logarithmic phase of growth. To maintain the cell morphology, the cells need to be fixed in methanol for 5 min and then to be permeabilized in cold acetone for 15 min to allow adequate penetration of serum antibodies (data not shown).

The diagnosis of SLE in canines involves several clinical and laboratory crite-

ria, such as a positive ANA test at high titers (>1:80) or a positive LE cell preparation confirmed by high ANA titers [5] [15].

The 50 samples used in this work were taken from cases of dogs clinically diagnosed with suspicion of various autoimmune diseases such as Lupus, Pemphigus, Myasthenia, autoimmune hemolytic anemia, and rheumatoid arthritis. However, only in 4 cases (id 1 to 4) the clinical diagnosis was discoid lupus erythematosus, which is the lupus-related disease most frequently diagnosed in Mexico [5] [15] [16].

The ANA International Consensus on Patterns (ICAP) recognizes 30 different patterns in HEp2 cells that have been associated with certain pathologies in human medicine [17]. The 30 IFA patterns in HEp2 cells are divided into 4 groups: negative (1), nuclear (15), cytoplasmic (9) and mitotic (5) patterns [8]. In contrast, in dogs, only five patterns have been described in HEp2 cells: the homogeneous pattern, with chromosomal staining of mitotic cells, the speckled pattern (fine and coarse) with non-chromosomal staining, perinuclear and nucleolar, however homogeneous, and mottled patterns are the most common. Several studies have attempted to correlate the activity of specific antibodies with patterns of nuclear labeling. Early studies had shown that anti-T1 (hnRNP G) gives a relatively fine speckle pattern, anti-Sm and anti-RNP give a coarser speckle or reticulum nodule pattern, and anti-histone antibody gives a generally homogeneous pattern [18].

The nuclear reaction patterns observed in MDCK cells can be explained by previously reported in samples from dogs with SLE, about of canine ANAs usually recognize histones or soluble nuclear antigens, whereas human ANAs react mainly with double-stranded DNA [19].

Therefore, the nuclear reaction pattern in MDCK correlates with antibodies against ds-DNA and DNA-associated proteins. A speckled pattern is associated with specific ANA, tRNP, Sm, SS-A, SS-B; also called extractable nuclear antigens (ENA) [20].

Likewise, the patterns observed in canine cells (MDCK) were different from those of Hep2 cells, probably because these cells contain different antigens in several of the proteins and histones associated with the nucleus and cytoplasm, in particular Histone 1 [21]. The above observation was verified using the SLE positive control serum included in the test kit for human, which in HEp2 cells, showed a strong homogeneous nuclear pattern, but in MDCK cells only a perinuclear pattern, with no nuclear reaction. Differences in the nuclear labeling pattern of HEp2 and MDCK were observed using sera from dogs that were also positive to the LE cell assay.

In our study, the strong positivity to the reaction of LE cells was associated with the diffuse (homogeneous) pattern in MDCK cells, (**Table 2**) in addition to the fact that this nuclear pattern is the one that has been most associated with systemic lupus erythematosus in dogs [1]. Likewise, the cases with negative LE cell test were negative for nuclear reaction in both cells ($p < 0.05$). It is well known that in humans, negativity to LE cells allows to rule out SLE with high

security, but positivity to LE cells correlates only in 50% to 75% of cases and is also found in other immune-mediated diseases [22]. These results mean that both tests would allow us to rule out SLE in canines.

Nuclear reactions occurred in MDCK could be associated with other types of canine Lupus or with dogs infected with *Ehrlichia canis* or *Bartonella vinsonii (berkhoffii)*. Furthermore, it has been observed that treatment with certain drugs such as griseofulvin, penicillin, sulfonamides, tetracyclines, phenytoin and procainamide can induce the development of ANA in some patients [5]. Therefore, the results obtained with MDCK cells suggest a greater sensitivity to detect other immune-mediated diseases.

The results when using fluorescence (HEp2) and peroxidase (MDCK) reactions were related at a high level, which agrees with the previous data of a high correlation between the determinations made by IFA-ANA and by EIA-ANA [23].

In the present work the test using HEp2 cells was considered as the gold test and the assay using MDCK cells was compared by contingency tables [24]. These results indicate that both tests are adequate to rule out the presence of antinuclear antibodies, but additional tests are required to confirm the disease [25].

6. Conclusions

Many questions remain to be answered regarding canine autoantigens and their correlation with different autoimmune diseases of connective tissue. Most canine autoantigens appear to differ from the major human autoantigens. The development of antigenic extracts designed for canine autoantigens is necessary to further develop canine diagnostic tools. A better understanding of canine ANAs and correlation with outcome appear necessary to further investigate and evaluate the nature of canine connective tissue autoimmune disorders (CTDs) [9].

The standardization of the two canine sera to be used as controls in the ANA tests by IP (**Table 3**) was very adequate since the Fleiss' Kappa concordance test ($p < 0.05$), yielded the following results among the three pathologists who performed the 10 replicate of two positive sera: Mild coarse granular (speckled pattern) (GG) 0.96 and moderate 0.92, moderate perinuclear pattern of 1.0 and severe fibrillar cytoplasmic of 1.0.

Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

No human or dog's patients were used in the present study, only blood samples submitted for diagnostic procedures from dogs.

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