

## Research paper

# Identification of specific antinuclear antibodies in dogs using a line immunoassay and enzyme-linked immunosorbent assay



Hanna D. Bremer<sup>a,\*</sup>, Erik Lattwein<sup>b</sup>, Stefanie Renneker<sup>b</sup>, Inger Lilliehöök<sup>c</sup>, Johan Rönnelid<sup>d</sup>, Helene Hansson-Hamlin<sup>a</sup>

<sup>a</sup> Department of Clinical Sciences, Swedish University of Agricultural Sciences (SLU), Box 7054, SE-750 07 Uppsala, Sweden

<sup>b</sup> Euroimmun AG, Lübeck, Germany

<sup>c</sup> Clinical Pathology Laboratory, University Animal Hospital, Swedish University of Agricultural Sciences, Uppsala, Sweden

<sup>d</sup> Department of Immunology, Genetics and Pathology, Uppsala University, Sweden

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## ABSTRACT

Circulating antinuclear antibodies (ANA) are commonly present in the systemic autoimmune disease Systemic Lupus Erythematosus (SLE) and in other systemic rheumatic diseases, in humans as well as in dogs. The indirect immunofluorescence (IIF)-ANA test is the standard method for detecting ANA. Further testing for specific ANA with immunoblot techniques or ELISAs is routinely performed in humans to aid in the diagnosis and monitoring of disease. Several specific ANA identified in humans have been identified also in suspected canine SLE but, in contrast to humans, investigation of autoantibodies in canine SLE is mainly restricted to the IIF-ANA test. Our aim was to identify both known and novel specific ANA in dogs and to investigate if different IIF-ANA patterns are associated with different specific ANA in dogs. Sera from 240 dogs with suspicion of autoimmune disease (210 IIF-ANA positive (ANA<sup>POS</sup>) and 30 IIF-ANA negative (ANA<sup>NEG</sup>)) as well as sera from 27 healthy controls were included. The samples were analysed with a line immunoassay, LIA (Euroline ANA Profile 5, Euroimmun, Lübeck, Germany) and four different ELISAs (Euroimmun). The ANA<sup>POS</sup> dogs were divided in two groups depending on the type of IIF-ANA pattern. Of the 210 ANA<sup>POS</sup> samples 68 were classified as ANA homogenous (ANA<sup>H</sup>) and 141 as ANA speckled (ANA<sup>S</sup>), one sample was not possible to classify. Dogs in the ANA<sup>H</sup> group had, compared to the other groups, most frequently high levels of anti-double stranded deoxyribonucleic acid (dsDNA) and anti-nucleosome ANA. Anti-dsDNA antibodies were confirmed in some dogs with the *Crithidia luciliae* indirect immunofluorescence test (CLIFT). The frequency of ANA<sup>H</sup> dogs with values above those observed in the healthy group was significantly higher compared to ANA<sup>S</sup> dogs for anti-dsDNA, anti-nucleosome, and anti-histone reactivity. Dogs in the ANA<sup>S</sup> group had, compared to the other groups, most frequently high levels of anti-ribonucleoproteins (RNP) and/or anti-Smith (Sm) antibodies. Reactivity against Sjögren's syndrome related antigens (SS)-A (including the Ro-60 and Ro-52 subcomponents), SS-B, histidyl tRNA synthetase (Jo-1), topoisomerase I antigen (Scl-70), polymyositis-scleroderma antigen (PM-Scl) and proliferating cell nuclear antigen (PCNA) was also noted in individual dogs. In conclusion, by using a commercial LIA and different ELISAs originally developed for detection of human ANA, we identified several specific ANA in serum samples from dogs sampled for IIF-ANA testing. Further, we found that the types of IIF-ANA pattern were associated with reactivity against some particular nuclear antigens.

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**Abbreviations:** ANA, antinuclear antibodies; ANA<sup>H</sup>, ANA homogenous; ANA<sup>NEG</sup>, IIF-ANA negative; ANA<sup>POS</sup>, IIF-ANA positive; ANA<sup>S</sup>, ANA speckled; CENP B, centromere protein B; CLIFT, *Crithidia luciliae* indirect immunofluorescence test; dsDNA, double stranded deoxyribonucleic acid; ENAs, extractable nuclear antigens; HEp-2, human epithelial-2; IIF, indirect immunofluorescence; Jo-1, histidyl tRNA synthetase; LIA, line immunoassay; M2, mitochondrial antigen 2; NSDTR, Nova Scotia Duck Tolling Retriever; PCNA, proliferating cell nuclear antigen; PM-Scl, polymyositis-scleroderma antigen; RIB, ribosomal P-protein; RNP, ribonucleoproteins; Scl-70, topoisomerase I; SLE, Systemic Lupus Erythematosus; Sm, Smith antigen; SS, Sjögren's syndrome related antigens.

\* Corresponding author.

E-mail address: [hanna.bremer@slu.se](mailto:hanna.bremer@slu.se) (H.D. Bremer).

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## 1. Introduction

Antinuclear antibodies (ANA) represent a heterogeneous group of autoantibodies directed at different nuclear antigens (Tan, 1989). Presence of high titres of ANA is a sensitive marker for the systemic autoimmune disease Systemic Lupus Erythematosus (SLE) in humans as well as in dogs (Tan et al., 1982; Bennett and Kirkham, 1987; Monier et al., 1992). Antinuclear antibodies, and some cytoplasmic autoantibodies, are also commonly present in other systemic rheumatic diseases (von Mühlen and Tan, 1995; Hansson-Hamlin et al., 2006; Hansson-Hamlin and Lilliehöök, 2009) as well as infrequently in healthy humans and dogs (Bennett and Kirkham, 1987; Tan et al., 1997). Positive titres of ANA can sometimes also be detected in canine leishmaniasis (Lucena et al., 1996), a disease that is rare in Sweden. The standard method for detecting ANA is indirect immunofluorescence; IIF (Coons et al., 1950; Tan et al., 1982; Bennett and Kirkham, 1987). In human diagnostics, human epithelial-2 (HEp-2) cells are commonly used as substrate (von Mühlen and Tan, 1995). In veterinary diagnostics, IIF-ANA test with HEp-2 cells are also used in the diagnosis of canine SLE and canine SLE-related disorders (Hansson et al., 1996; Bell et al., 1997).

Antinuclear antibodies are directed against several different antigens or groups of antigens such as double stranded deoxyribonucleic acid (dsDNA), histones, Smith antigen (Sm), ribonucleoproteins (RNP), Sjögren's syndrome related antigens; (SS)-A/Ro, and SS-B/La (Tan, 1989). Several specific ANA previously identified in humans like the anti-histone (Costa et al., 1984; Monier et al., 1992), anti-RNP (Monier et al., 1978, 1980; Costa et al., 1984; Thoren-Tolling and Ryden, 1991; Fournel et al., 1992; Welin Henriksson et al., 1998; Hansson-Hamlin and Rönnelid, 2010), anti-Sm (Monier et al., 1978; Costa et al., 1984; Hubert et al., 1988; Fournel et al., 1992), anti-SS-A (Monier et al., 1988; Fournel et al., 1992), and anti-SS-B (Monier et al., 1992) have been identified also in suspected canine SLE cases. In addition, specific ANA against a 43 kDa protein known as the hnRNP G and against an unidentified antigen called type-2 antigen have been identified in dogs but not in humans (Costa et al., 1984; Soulard et al., 1991; Fournel et al., 1992). Anti-dsDNA antibodies in canine SLE have only infrequently been reported by some authors (Fournel et al., 1992; Monier et al., 1992).

Different IIF patterns are indicative of specific ANA (von Mühlen and Tan, 1995). Two common IIF patterns in humans are the homogenous pattern and the speckled pattern. Other patterns are also recognised. 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 RNP, Sm, SS-A, SS-B; also called extractable nuclear antigens; ENAs (Sharp et al., 1972; Tozzoli et al., 2002). The IIF-pattern and specific ANA can also be associated with different autoimmune disorders, some with high specificity for a particular disease (von Mühlen and Tan, 1995). For example, the anti-dsDNA and anti-Sm antibodies are quite specific for human SLE, and included in the diagnostic criteria for human SLE (Tan et al., 1982; Hochberg, 1997). Some specific ANA, like the anti-dsDNA antibodies, are associated with particular symptoms and can correlate with disease activity (Koffler et al., 1967; Swaak et al., 1986; Terborg et al., 1990). Hence, testing for specific ANA is an aid in the diagnosis and monitoring of autoimmune diseases in humans. Commonly used methods to screen for specific ANA in humans are ELISA and immunoblot techniques (Tozzoli et al., 2002; Murdjeva et al., 2011). In order to investigate anti-dsDNA antibodies, the *Crithidia luciliae* indirect immunofluorescence test (CLIFT) is also a commonly used method (Aarden et al., 1975).

In contrast to humans where testing for specific ANA is routinely performed in suspected SLE cases, investigation of autoantibodies in suspected canine SLE is mainly restricted to the IIF-ANA test

(Bennett, 1987). The IIF-ANA test is almost always positive in canine SLE, but a positive test alone is not sufficient for the diagnosis. Therefore, other clinical, haematological and/or biochemical alterations also have to be present (Bennett, 1987). In dogs, two distinct immunofluorescence patterns can be identified when using HEp-2 cells as a substrate (Fig. 1), the homogenous pattern, with chromosomal staining of mitotic cells and the speckled pattern, with non-chromosomal staining (Hansson and Karlsson-Parra, 1999). The different patterns have been correlated with clinical signs in dogs, suggesting that dogs with a homogenous pattern more often show involvement of several organ systems than dogs with a speckled pattern (Hansson-Hamlin et al., 2006).

Today, even though there is some knowledge about specific ANA in dogs, previous studies show that more research is needed in the field. The occurrence of some specific ANA that are of importance in human medicine has not been investigated in dogs. Unidentifiable ANA have also been found in dogs (Monier et al., 1980; Costa et al., 1984; Hansson and Karlsson-Parra, 1999). Further, very little is known about the clinical and pathological role of specific ANA in dogs. In the present study we investigated sera from IIF-ANA tested dogs for specific ANA reactivity against several antigens known to be associated with systemic autoimmune or rheumatic disease in humans. Our aim was to identify both known and novel specific ANA in dogs and to investigate if different IIF-ANA patterns are associated with different specific ANA in dogs.

## 2. Materials and methods

### 2.1. Study design and study population

Included in the study were 210 IIF-ANA positive (ANA<sup>pos</sup>) and 30 IIF-ANA negative (ANA<sup>neg</sup>) dog sera. The samples had been submitted to the Clinical Pathology Laboratory, University Animal Hospital, Uppsala, Sweden, from veterinarians all over Sweden for routine IIF-ANA testing during May 2002–June 2012. Eight of the dogs had been resampled 35–480 days from the first sampling occasion, so for these dogs two serum samples were available. For the majority of dogs included in the study, clinical information was not available, but the ANA<sup>pos</sup> as well as the ANA<sup>neg</sup> dogs were assumed to have a suspicion of autoimmune disease since the samples had been sent for IIF-ANA testing.

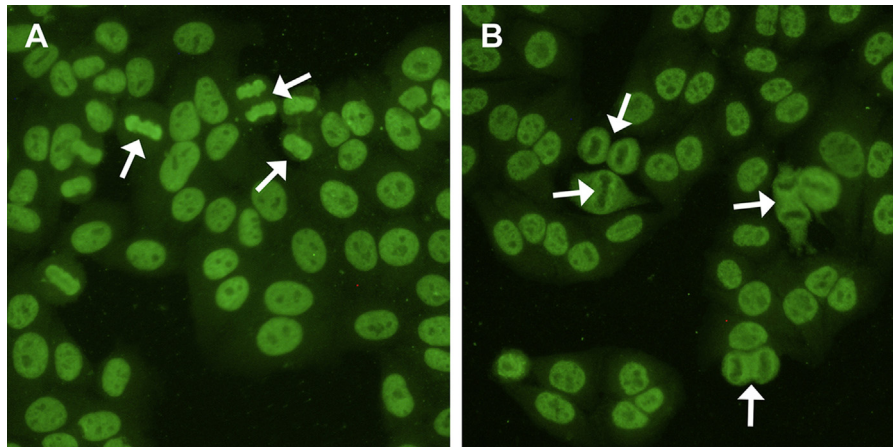
The sera had been stored in  $-20$  or  $-70$  °C until analysis and had been through two to four thaw–freeze cycles. The serum samples were sent to Euroimmun, Lübeck, Germany, where the IIF-ANA tests were repeated. In order to detect specific ANA, two different assays were performed, a line immunoassay (LIA) and an ELISA. As a healthy control group, sera from 27 blood donor dogs and laboratory dogs from Germany were also analysed. No information about sex and breed was available for these dogs. In total, 275 serum samples from 267 dogs were investigated for IIF-ANA and for the presence of specific ANA.

Confirmatory analyses of ds-DNA reactivity with CLIFT were performed in 39 of the diseased dog sera as well as in 12 healthy control sera (collected from the Canine Biobank, SLU, Uppsala, ethical permission C2/12).

The blood samples from the diseased dogs were initially taken for diagnostic purposes (IIF-ANA tests) and for health screening for the control dogs. An ethical permission was obtained from the local ethical committee, Uppsala, Sweden (C 418/12).

### 2.2. IIF-ANA test

IIF-ANA tests were performed at the Clinical Pathology Laboratory, University Animal Hospital, Swedish University of Agricultural



**Fig. 1.** Picture illustrating two distinct immunofluorescence patterns identified in dog sera with human epithelial-2 cells as substrate. (A) Homogenous antinuclear antibody (ANA) pattern. Arrows are pointing at mitotic cells representing positive staining of chromosomal regions. (B) Speckled ANA pattern. Arrows are pointing at mitotic cells showing a negative staining of chromosomal regions surrounded by positive nucleosome staining.

Sciences and at Euroimmun (Lübeck, Germany). Monolayers of HEp-2 cells fixed on glass slides (Immuno Concepts, Sacramento, CA, USA and Euroimmun, respectively) were incubated with sera according to the manufacturer's instructions as described previously (Hansson et al., 1996). The glass slides were examined by fluorescence microscopy and considered positive at a titre of  $\geq 1:100$ . The visible nuclear fluorescence pattern was divided into two groups; homogenous (ANA<sup>H</sup>) or speckled (ANA<sup>S</sup>) patterns as previously described (Hansson et al., 1996; Hansson-Hamlin et al., 2006). The samples from dogs with suspicion of autoimmune disease (ANA<sup>POS</sup> and ANA<sup>NEG</sup> dogs) were analysed for IIF-ANA positivity both in Sweden and in Germany, the samples from the healthy control dogs were only analysed in Germany. The type of IIF-ANA pattern was only reported by Euroimmun.

### 2.3. Line immunoassay (Euroline ANA Profile 5)

All the samples were analysed with the Euroline ANA Profile 5 (Euroimmun) which is a line immunoassay (LIA), a type of immunoblot, where recombinant antigens (RNP-A, RNP-C, RNP-70, SS-A/Ro-52, PM-Scl, centromere protein B (CENP B), proliferation cell nuclear antigen (PCNA)) and native antigens (dsDNA, nucleosomes, histones, nRNP/Sm, Sm, SS-A/Ro-60, SS-B/La, DNA topoisomerase I (Scl-70), histidyl tRNA synthetase (Jo-1), mitochondrial antigen 2 (M2), ribosomal P-proteins (RIB)) are coated as discrete lines on membrane chips with a plastic backing (Schlumberger et al., 1995). Additionally one control line is coated on each strip. The assay was performed according to the manufacturer's instructions with the exception of the anti-human-IgG conjugate, which was exchanged for alkaline phosphatase-conjugated rabbit anti-dog IgG (Euroimmun). Briefly, the test sample was diluted 1:101 in the sample buffer provided. If specific canine autoantibodies were present in the test serum, they bound to the antigen containing lines on the strip. Anti-canine IgG then bound to any previously formed antigen/antibody complex. Incubation with chromogen Nitroblue Tetrazolium Chloride/5-Bromo-4-chloro-3-indolylphosphate (NBT/BCIP) produced a dark blue colour proportional to the amount of specific antibody present in the test sample. Colour development was stopped by adding distilled water after having discharged the substrate solution. The strips were evaluated by automated examination using a flatbed scanner and the EuroLineScan software (Euroimmun) and quantification was performed based on the colour intensity.

### 2.4. ELISA tests

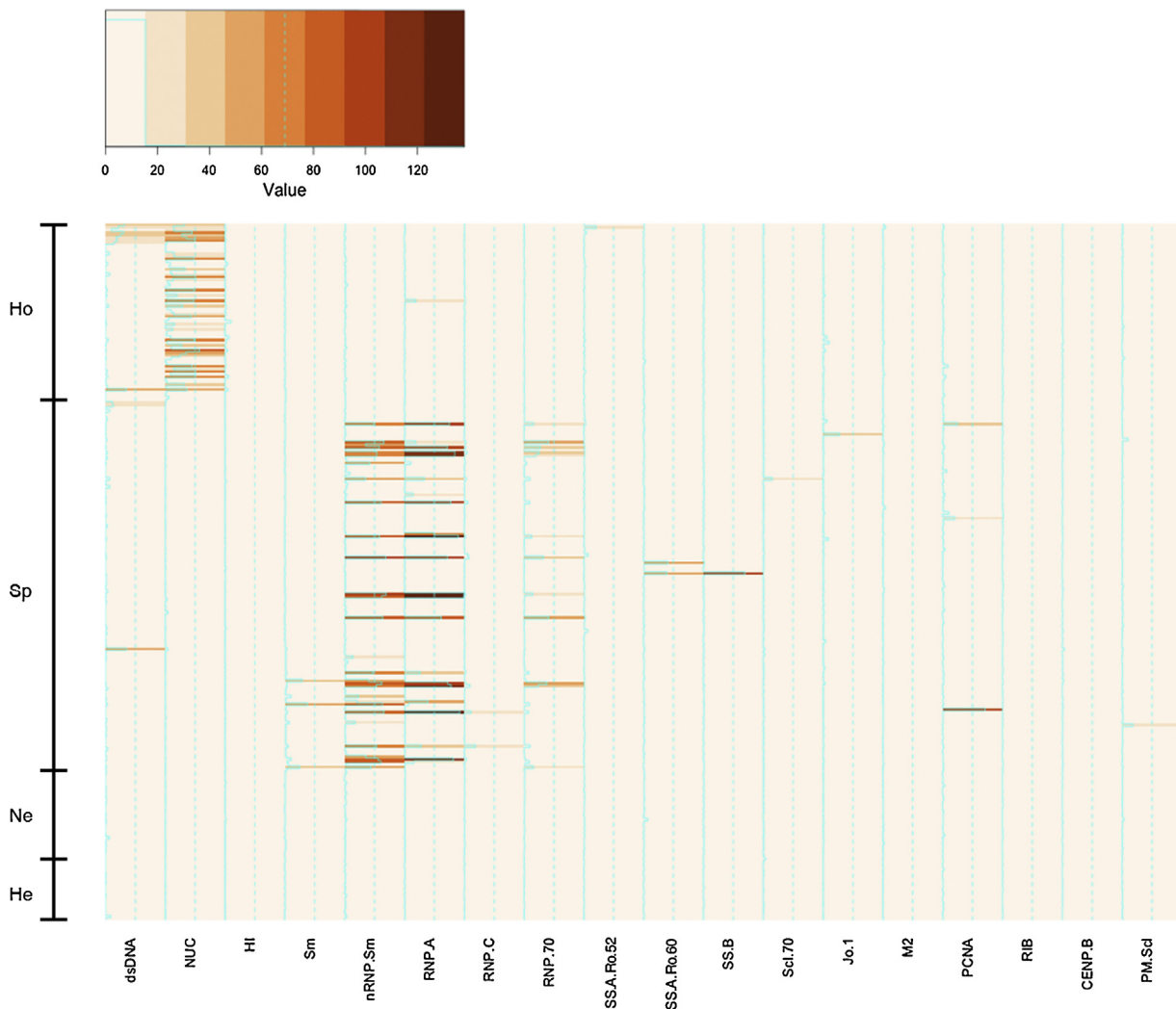
The serum samples were analysed in a 1:201 dilution with four different ELISAs (Euroimmun). The ELISAs used were the anti-dsDNA ELISA for detection of autoantibodies against double-stranded genomic DNA, the anti-nucleosomes ELISA for the detection of autoantibodies against nucleosomes, the anti-dsDNA-NcX ELISA for the detection of autoantibodies against dsDNA complexed with nucleosomes, and the anti-ENA ProfilePlus 1 ELISA for the detection of antibodies against six different ENAs, namely nRNP/Sm, Sm, SS-A/Ro-60, SS-B, Scl-70 and Jo-1. All assays were performed according to the manufacturer's instructions with the exception of the anti-human-IgG conjugate, which was exchanged for peroxidase-conjugated rabbit anti-dog IgG (Euroimmun). Photometric measurement of the colour intensity was performed using an automated reader (TECAN) in combination with Magellan software.

### 2.5. CLIFT

Sera positive for anti-dsDNA with ELISA or LIA were further investigated with the CLIFT at the Department of clinical immunology at Uppsala University Hospital. Samples were diluted 1:10, and staining was performed on *C. luciliae* substrate (ImmunoConcept) according to the hospital laboratory protocol at Uppsala University Hospital intended for human patients, but with the exchange of the anti-human conjugate to the fluorescein isothiocyanate-coupled rabbit anti-dog IgG antibody (Sigma-Aldrich, Stockholm, Sweden) used in the IIF-ANA test described above, and with the same dilution (1:100) as used in the IIF-ANA test. The microscope slides were evaluated by an experienced interpreter (JR).

### 2.6. Statistical analysis

Data were assembled using Microsoft® Excel® 2011, version 14.4.5 and software R version 3.0.2 (R Foundation for Statistical Computing). Statistical analyses were performed using Vassarstats (<http://vassarstats.net/odds2x2.html>) and software R. To compare the ANA<sup>H</sup> and the ANA<sup>S</sup> groups for specific ANA, we used the highest observed value in the healthy group as a cut-off value and compared the frequency of positive cases between the groups with the Fisher exact probability test (two-tailed). Kendall's *tau* ( $\tau$ ) was calculated to assess the degree of correlation between the LIA and ELISA assays using R package stats (v. 3.0.2). *p*-Values less than 0.05 were considered as significant.



**Fig. 2.** Heat map of antibody levels against all the antigens in the line immunoassay (LIA) for all the dogs included in the study ( $n = 266$ ). Ho, antinuclear antibody (ANA) positive with a homogenous pattern ( $n = 68$ ); Sp, ANA positive with a speckled pattern ( $n = 141$ ); Ne, ANA negative ( $n = 30$ ); He, healthy dogs ( $n = 27$ ).

### 3. Results

#### 3.1. IIF-ANA test

The IIF-ANA test results (positive/negative) obtained from Sweden were confirmed in all the samples at repeated analyses in Germany. Of the 210 serum samples from ANA<sup>pos</sup> dogs that were analysed with regards to IIF-ANA pattern and ANA specificity, 68 were classified as ANA<sup>H</sup>, 141 as ANA<sup>S</sup> and one sample was not possible to classify as ANA<sup>H</sup> or ANA<sup>S</sup> and was therefore excluded from further analyses. In the healthy control group, one serum sample was ANA positive with a speckled pattern. In total 266 canine sera from 239 dogs with suspicion of autoimmune disease (68 ANA<sup>H</sup>, 141 ANA<sup>S</sup>, 30 ANA<sup>neg</sup>) and from 27 healthy dogs were evaluated and compared with regards to specific ANA reactivity. The breed and sex distribution in the three ANA groups are presented in Table 1. Note that the healthy group is not included in the table, since information about sex and breed was not available. Sera from eight dogs that were sampled twice, having had 35 to 480 days in between, showed the same IIF-ANA pattern at both occasions.

#### 3.2. Specific antigen reactivity

Specific ANA reactivities measured in the LIA in individual dogs are presented as a heat map (Fig. 2), which allows the ability to see

combinations of reactivities in individual dogs and dogs with similar ANA specificity. A heat map for the ELISA results is presented as supplementary material (Figure S1). Findings of particular interest are discussed in the following sections.

##### 3.2.1. Antibodies to dsDNA, nucleosomes, and histones

The results from the LIA and the ELISAs for dsDNA, nucleosomes and histones for the four different groups of dogs are presented in Fig. 3. Dogs in the ANA<sup>H</sup> group had, compared to the other groups, most frequently high levels of anti-dsDNA and anti-nucleosome ANA (measured as OD and band intensity in the ELISAs and LIA, respectively). The number of dogs in the ANA<sup>H</sup> group with anti-dsDNA reactivity above that observed in the healthy group was 9 (13%) in the LIA and 22 (32%) in the ELISA, respectively. This is a significant difference compared to the ANA<sup>S</sup> group (Fisher's test  $p = 0.002$  for LIA,  $p < 0.001$  for ELISA) where the corresponding number of dogs were 3 (2.1%) in the LIA and 15 (11%) in the ELISA. In total sera from 39 dogs (22 ANA<sup>H</sup>, 16 ANA<sup>S</sup>, one ANA<sup>neg</sup>) had anti-dsDNA reactivity in the LIA and/or the ELISA. Of these, 10 sera (9 ANA<sup>H</sup>, one ANA<sup>S</sup>) were positive against dsDNA with CLIFT (Fig. 4). All the 12 healthy control samples were negative.

The number of dogs in the ANA<sup>H</sup> group with anti-nucleosome reactivity above that observed in the healthy group was 59 (87%) in the LIA and 64 (94%) in the ELISA, a significant difference compared to the ANA<sup>S</sup> group ( $p < 0.001$  for LIA and ELISA) where the

**Table 1**  
Number of dogs (%) of each breed and sex distributed according to the three main ANA patterns.

|                     | ANA <sup>neg</sup> | ANA <sup>H</sup> | ANA <sup>S</sup> | Total     |
|---------------------|--------------------|------------------|------------------|-----------|
| German Shepherd Dog | 3 (10)             | 4 (5.9)          | 26 (18)          | 33 (14)   |
| NSDTR               | 2 (6.7)            | 17 (25)          | 13 (9.2)         | 32 (13)   |
| Cocker Spaniel      | 0 (0.0)            | 2 (2.9)          | 16 (11)          | 18 (7.5)  |
| Mixed-breed         | 0 (0.0)            | 1 (1.5)          | 11 (7.8)         | 12 (5.0)  |
| Golden Retriever    | 2 (6.7)            | 2 (2.9)          | 7 (5.0)          | 11 (4.6)  |
| Cairn Terrier       | 1 (3.3)            | 5 (7.4)          | 2 (1.4)          | 8 (3.3)   |
| Jack Russel         | 1 (3.3)            | 4 (5.9)          | 1 (0.7)          | 6 (2.5)   |
| Others              | 21 (70)            | 33 (49)          | 65 (46)          | 119 (50)  |
| Total               | 30 (100)           | 68 (100)         | 141 (100)        | 239 (100) |
| Females             | 12 (40)            | 45 (66)          | 66 (47)          | 123 (51)  |
| Males               | 18 (60)            | 22 (32)          | 74 (52)          | 114 (48)  |
| Unknown             | 0 (0.0)            | 1 (1.5)          | 1 (0.71)         | 2 (0.84)  |

All dogs were suspected to have autoimmune disease. Breeds with  $n \geq 5$  presented as individual breeds, dogs of other breeds ( $n < 5$ ) and unknown breeds ( $n = 9$ ) presented as others.

Information about neutering inconsistently reported, neutered animals therefore included in females and males. Healthy dogs ( $n = 27$ ) not included (see text).

ANA, antinuclear antibody; ANA<sup>neg</sup>, antinuclear antibody negative; ANA<sup>H</sup>, antinuclear antibody homogenous; ANA<sup>S</sup>, antinuclear antibody speckled; NSDTR, Nova Scotia Duck Tolling Retriever.

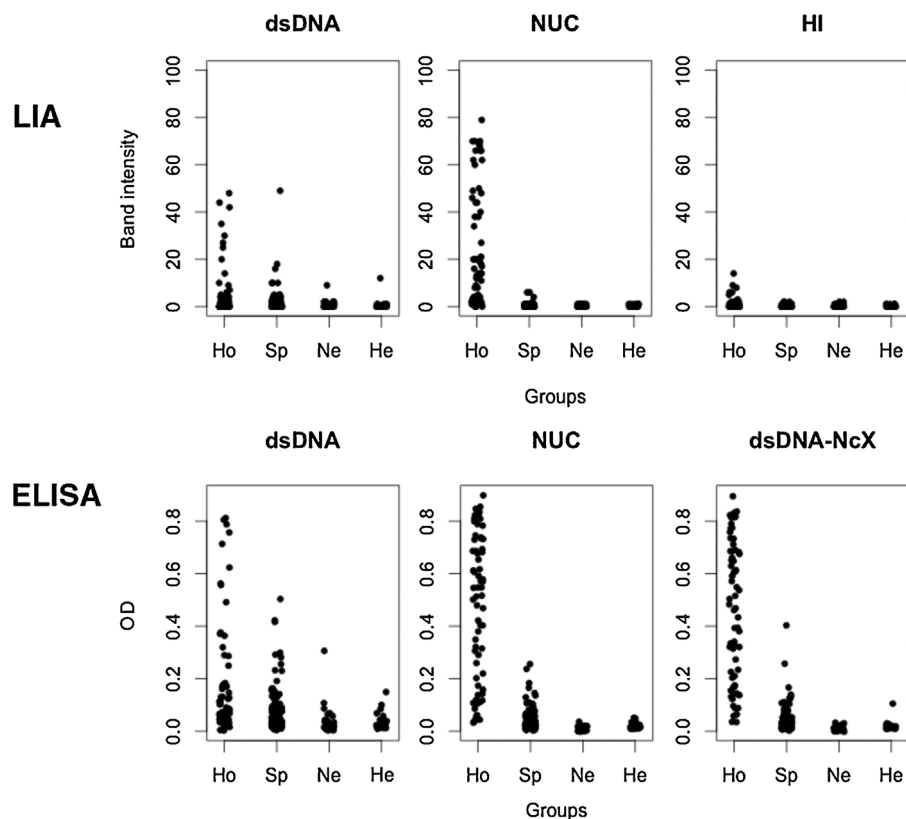
corresponding number of dogs was 4 (2.8%) in the LIA and 35 (25%) in the ELISA. The frequency of ANA<sup>H</sup> dogs with values above those observed in the healthy group was higher compared to ANA<sup>S</sup> dogs both for anti-histone reactivity ( $p < 0.001$  for LIA) and anti-dsDNA-NcX reactivity ( $p < 0.001$  for ELISA). All dogs in the ANA<sup>H</sup> group that had anti-dsDNA reactivity (defined as value above that observed in the healthy group) also had anti-nucleosome reactivity (Figs. 2 and S1).

The values from the ELISA and LIA plotted against each other for dsDNA, nucleosomes and the other antigens that were present in both assays are provided as supplementary material (Figure S2).

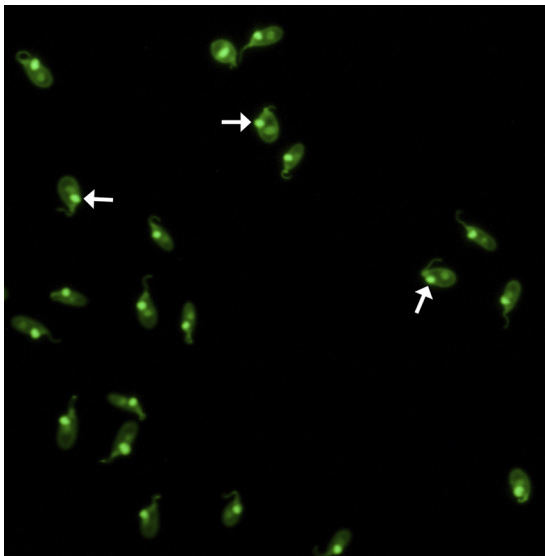
### 3.2.2. Antibodies to extractable nuclear antigens

The results from the LIA and ELISAs for the extractable nuclear antigens RNP (RNP-A, RNP-C, RNP-70), Sm, nRNP/Sm, SS-A/Ro-60, SS-A/Ro-52, SS-B, Scl-70 and Jo-1 are presented in Fig. 5.

Dogs in the ANA<sup>S</sup> group had, compared to the other groups, most frequently high levels of anti-RNP and/or anti-Sm antibodies. The number of dogs in the ANA<sup>S</sup> group with anti-nRNP/Sm reactivity above that observed in the healthy group was 32 (23%) in the LIA and 36 (26%) in the ELISA, a significant difference compared to the ANA<sup>H</sup> group ( $p < 0.001$  for LIA and ELISA) where the corresponding number of dogs was 1 (1.5%) in both the LIA and the ELISA. Comparing the frequency of dogs with values above that observed in the



**Fig. 3.** Antibody reactivity against dsDNA, nucleosomes (NUC), histones (HI) and dsDNA complexed with NUC (dsDNA-NcX). Results from line immunoassay (LIA) and ELISA presented. Note that the some of the antigens vary between the assays. Ho, antinuclear antibody (ANA) positive with a homogenous pattern; Sp, ANA positive with a speckled pattern; Ne, ANA negative; He, healthy dogs.



**Fig. 4.** Indirect immunofluorescence staining of *Crithidia luciliae* incubated with dog serum, illustrating positive anti-dsDNA reactivity. Arrows are pointing at staining of mitochondrial DNA.

healthy group for the remaining ENAs, the frequency was higher in the ANA<sup>S</sup> group than in the ANA<sup>H</sup> group for RNP-A ( $p < 0.001$  for LIA), RNP-C ( $p = 0.007$  for LIA), RNP-70 ( $p < 0.001$  for LIA) and Sm ( $p < 0.001$  for LIA,  $p = 0.002$  for ELISA). There was no difference in frequency between the ANA<sup>H</sup> and ANA<sup>S</sup> group for the remaining antigens but some dogs had reactivities above that observed in the healthy groups against SS-A, SS-B, Jo-1, Scl-70, and individual dogs had a particularly strong reactivity (Fig. 5). One dog had strong reactivity for anti-SS-A as well as anti-SS-B antibodies measured both with LIA and ELISA (Figs. 2, 5, S1 and S2). Another dog had strong anti-SS-A reactivity confirmed by both methods, while yet another dog had single SS-A reactivity only measured in the ELISA but not confirmed in the LIA (Figure S2). Antibodies against Sm and RNP antibodies were often observed in the same individuals (Figs. 2 and S1).

### 3.2.3. Antibodies to remaining antigens

Antibodies against M2, PCNA, RIB, CENP-B and PM-Scl, were only measured with the LIA. The results are presented in Fig. 6. Individual dogs in the ANA<sup>S</sup> group had clear anti-PM-Scl or anti-PCNA reactivity.

## 4. Discussion

With the use of human commercial LIA and ELISA tests we identified several specific ANA in serum samples from dogs sampled for IIF-ANA testing. Further, we found that the reactivity against specific nuclear antigens was associated with individual IIF-ANA patterns. Anti-dsDNA and anti-nucleosome antibodies were most frequently observed in the ANA<sup>H</sup> group which is in agreement with observations in humans (von Mühlen and Tan, 1995). Findings of anti-DNA and anti-nucleosome antibodies also in some dogs with an IIF-ANA speckled pattern might indicate that these dogs actually have a mixed IIF-ANA pattern.

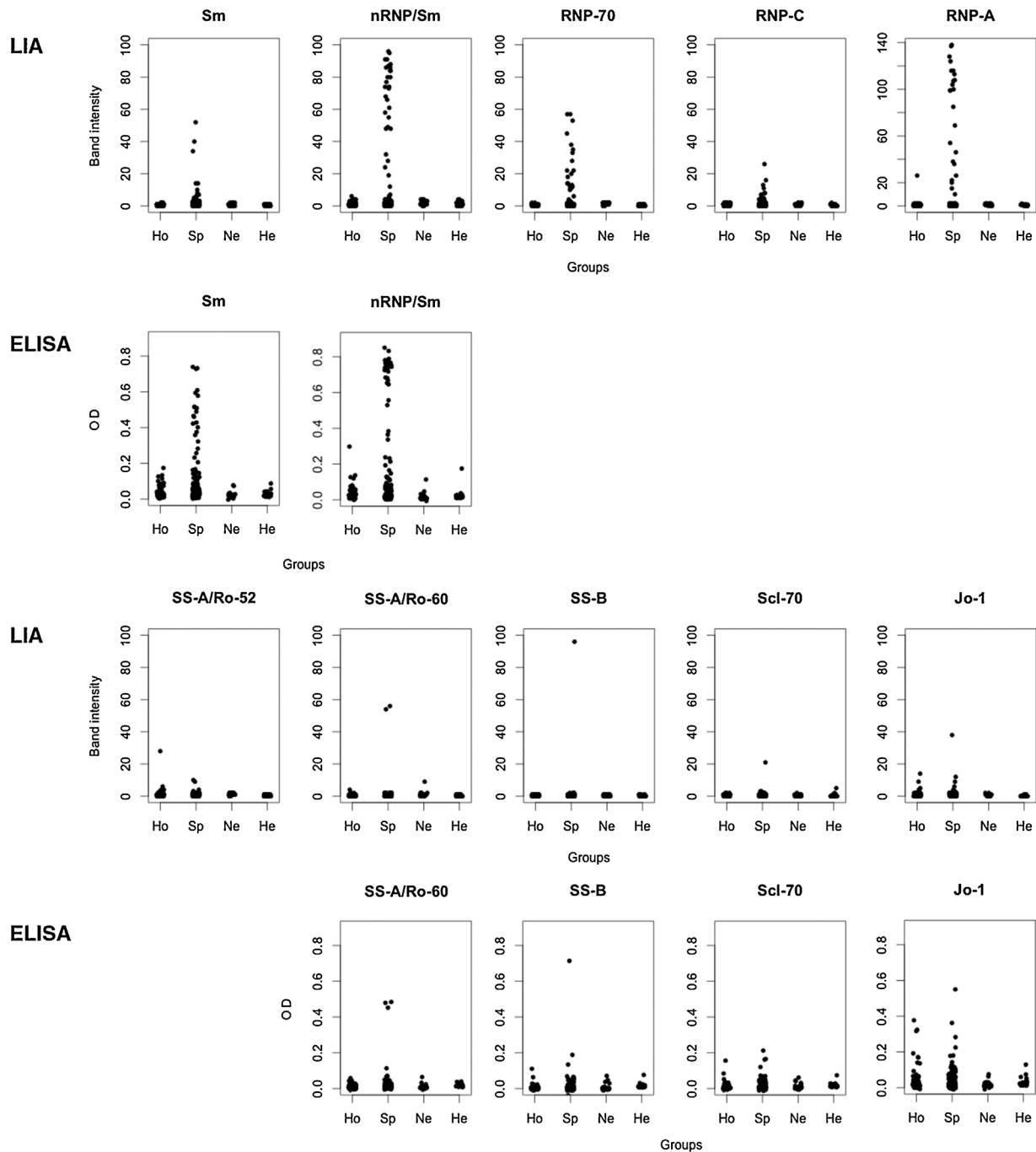
Previous investigations of anti-dsDNA antibodies in dogs have yielded inconclusive results. While some authors have identified anti-dsDNA antibodies (Fournel et al., 1992; Monier et al., 1992), others have investigated a large number of ANA positive dogs for anti-dsDNA antibodies with negative results (Monestier et al., 1995; Hansson and Karlsson-Parra, 1999).

We could confirm anti-dsDNA reactivity with CLIFT in 10 out of 39 sera that were positive in the ELISA and/or the LIA. In sera with a homogenous pattern in the HEp-2 IIF, 9 out of 22 were CLIFT positive. The CLIFT staining pattern was exactly the same as with human SLE sera. The CLIFT has previously been applied in canine studies, mostly with negative results (Brinet et al., 1988; Hansson and Karlsson-Parra, 1999), but Fournel et al. (1992) detected anti-dsDNA antibodies in two dogs with CLIFT. In our study the five samples with highest antibody levels in the ELISA were confirmed to be anti-dsDNA positive by CLIFT, however, there was no clear correlation between the CLIFT result and the antibody levels in the ELISA and the LIA for the rest of the samples. It is well known from human studies, that there is a considerable discordance between anti-dsDNA detection methods (Haugbro et al., 2004; Enocsson et al., 2015). Different assays can detect different subpopulations of anti-dsDNA autoantibodies with different avidity. ELISAs also detect low avidity anti-dsDNA antibodies, which can occur in other diseases than SLE. There are additional explanations for discrepant results in different assays, for example that the source and the presentation of the antigens vary between assays, and that the reaction conditions differ (Smeenk, 2002; Riboldi et al., 2005). Consequently, it is not surprising that we could not confirm anti-dsDNA reactivity in all dogs. CLIFT has lower sensitivity than enzyme immunoassays, but is highly specific for anti-dsDNA reactivity in SLE patients (Haugbro et al., 2004; Enocsson et al., 2015).

The majority of dogs with anti-dsDNA reactivity also had anti-nucleosome reactivity and reactivity against dsDNA complexed with nucleosomes. Monestier et al. (1995) previously investigated reactivity against nucleosomes in 43 dogs with SLE and found no significant difference in anti-nucleosome reactivity between SLE and control dogs. However the mean value in the SLE dogs was slightly higher than in controls, indicating that although not significant at group level, individual SLE dogs might have had high anti-nucleosome antibody levels. Anti-nucleosome antibodies have been suggested to be a better diagnostic marker than anti-dsDNA antibodies in human SLE (Bizzaro et al., 2012) and the ELISA with dsDNA complexed with nucleosomes might be superior to anti-dsDNA and anti-nucleosome ELISAs (Biesen et al., 2011). Our results show that many ANA positive dogs, mainly ANA<sup>H</sup> dogs, have these antibodies, but if any of these assays can be used in the diagnosis of canine SLE needs to be further investigated.

Previous work in dogs have reported anti-histone antibodies to be common in canine SLE (Costa et al., 1984; Monier et al., 1992; Monestier et al., 1995) and therefore have been suggested to be included in the criteria for canine SLE (Chabanne et al., 1999). In comparison, we observed anti-histone antibodies in some ANA<sup>POS</sup> dogs, but at a lower frequency. Since clinical information was not available for the dogs in our study it is not possible to know how many of the canine patients suffered from SLE. This might explain the lower frequency compared to studies including only SLE dogs. Furthermore, antigens and exposure of different epitopes might vary between assays, a circumstance that might influence the results.

Antibodies against RNP and Sm were mostly present in the ANA<sup>S</sup> group, also in agreement with observations in humans (von Mühlen and Tan, 1995; Wiik et al., 2010). A few dogs in the ANA<sup>H</sup> group had anti-RNP and anti-Sm reactivity, but they also had anti-nucleosome reactivity, which explains the homogenous pattern. In agreement with human observations (Sherer et al., 2004), anti-RNP and anti-Sm antibodies were often present together in the same dog. Anti-Sm antibodies have high specificity for human SLE, while antibodies against RNP without anti-Sm can be associated with mixed connective tissue disease (Sharp et al., 1972) but might also occur in other systemic rheumatic diseases like myositis and systemic sclerosis. Both anti-RNP and anti-Sm antibodies have previously been identified in dogs (Costa et al., 1984; Hubert et al.,



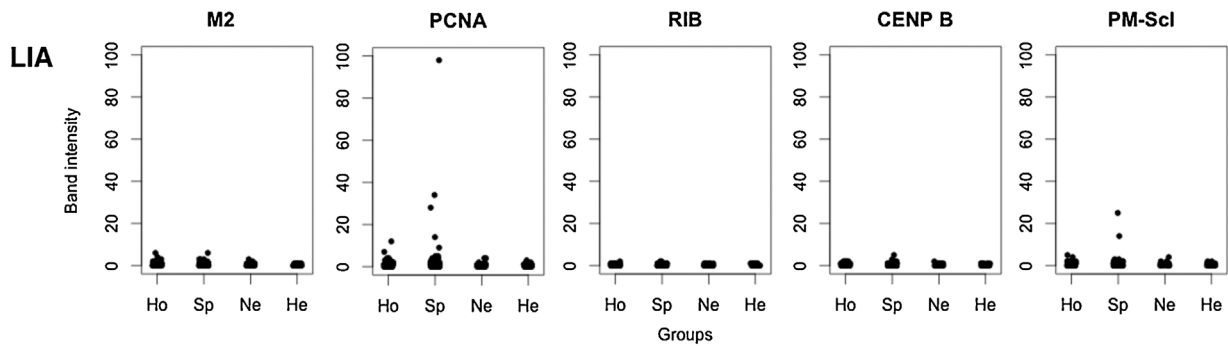
**Fig. 5.** Antibody reactivity against extractable nuclear antigens (ENAs). Results from line immunoassay (LIA) and ELISA presented. Note that the some of the antigens vary between the assays. Ho, antinuclear antibody (ANA) positive with a homogenous pattern; Sp, ANA positive with a speckled pattern; Ne, ANA negative; He, healthy dogs.

1988; Fournel et al., 1992; Welin Henriksson et al., 1998; Hansson-Hamlin and Rönnelid, 2010). Specific ANA that could be detected in only a few dogs were anti-SS-A, anti-SS-B, anti-Scl-70, anti-Jo1 and anti-PCNA. To our knowledge, specific ANA against Scl-70 and antibodies to the cytoplasmic antigen Jo-1 have not previously been identified in dogs. Anti-Scl-70 antibodies are associated with systemic sclerosis (Shero et al., 1986) and anti-Jo-1 with polymyositis (Nishikai and Reichlin, 1980) in humans. Suspected anti-PCNA antibodies have previously been reported in one dog with SLE-related disease (Goudswaard et al., 1993). In humans, anti-PCNA antibodies are associated with, but not specific for SLE (Mahler et al., 2012). Human studies have indicated that multiple reactivities are more common in SLE than other SLE-related disorders and can also be

associated with more severe SLE disease (Notman et al., 1975; Boey et al., 1988). In our study we observed that some dogs had high antibody levels against several antigens, but the clinical significance of this is not known.

It was not possible to detect the specific ANA in all the ANA<sup>POS</sup> dogs. One reason could be that the 43 kDa protein known as hnRNP-G (Costa et al., 1984; Soulard et al., 1991) and the type-2 antigen (Costa et al., 1984; Fournel et al., 1992), which are thought to be important canine antigens, were not included in the assays.

Several of our results are in agreement with previous work about ANA specificity in dogs. However, comparing frequency of positive cases between studies is not possible, since the inclusion criteria, methods and interpretation of positive samples vary a lot between



**Fig. 6.** Antibody reactivity against M2, PCNA, RIB, CENP B and PM-Scl in the line immunoassay (LIA). Ho, antinucler antibody (ANA) positive with a homogenous pattern; Sp, ANA positive with a speckled pattern; Ne, ANA negative; He, healthy dogs.

studies. Here we used the highest observed value in the healthy group as a cut-off to compare the two ANA<sup>POS</sup> groups. It should be noted that the distribution of age, sex, and breeds of the healthy dogs was not known so this group might not be well representative of the diseased group. We would not recommend a cut-off for a clinical test being based on the results presented here. To establish a cut-off was not the aim of our study but instead to discover high values of specific ANA in individual dogs. Even if a large representative control group was to be analysed, establishing a cut-off for a clinical test would be a challenge, especially for the LIA, because the data are not normally distributed and not entirely continuous. The LIA is semi-quantitative, and small differences in observed values might be of little importance.

ELISA and LIA are routinely used in human diagnostics for the detection of specific autoantibodies. Both techniques are quantitative (ELISA) or at least semi-quantitative (LIA), automatable and therefore suitable for large scale testing. The evaluation is performed using special software making it easier to obtain standardised results. Up to now, no such techniques are used routinely for ANA diagnostics in dogs but both might be adapted in the future. However, both assays are prototypes and have to be validated and adjusted accordingly. In a few dogs the ELISA detected high levels of antibodies that were not confirmed with the LIA. The reason for this is not obvious but ELISAs are known to be very sensitive in detecting antibodies, but to have lower specificity compared to other methods, while immunoblot techniques sometimes fail to detect antibodies against some antigens like SS-A and Scl-70 (Phan et al., 2002; Orton et al., 2004). This is a likely explanation to the weak agreement observed for these two antigens in our study.

Further research is also needed to relate the results for specific antigens in ELISA and LIA to clinical findings. The lack of clinical data for the majority of dogs is a limitation of our study. Clinical records were available for some of the dogs, but there were too few dogs to speculate about a connection between specific ANA and clinical signs. Since specific ANA are associated with particular autoimmune diseases and symptoms in humans, finding similar associations in dogs would be very useful. There is a need for more specific tests for autoimmune diseases in dogs, and future research should aim at correlating specific ANA with particular clinical signs.

## 5. Conclusion

We investigated sera from a large number of dogs for specific ANA with commercial assays originally developed for the detection of human ANA. Several specific ANA previously described in dogs were identified, confirming results from previous studies. In individual dogs we also identified ANA of importance in human medicine but not previously described in dogs. Further, different types of IIF-ANA patterns were shown to be associated with specific ANA reactivities.

## Conflict of interest statement

Erik Lattwein and Stefanie Renneker are employees of Euroimmun, Lübeck, Germany. Euroimmun performed the laboratory analyses except where otherwise stated.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vetimm.2015.10.002>.

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