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Research paper

Canine susceptibility to visceral leishmaniasis: A systematic review upon genetic aspects, considering breed factors and immunological concepts

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ABSTRACT

Dogs have different susceptibility degrees to leishmaniasis; however, genetic research on this theme is scarce, manly on visceral form. The aims of this systematic review were to describe and discuss the existing scientific findings on genetic susceptibility to canine leishmaniasis, as well as to show the gaps of the existing knowledge. Twelve articles were selected, including breed immunological studies, genome wide associations or other gene polymorphism or gene sequencing studies, and transcription approaches. As main results of literature, there was a suggestion of genetic clinical resistance background for Ibizan Hound dogs, and alleles associated with protection or susceptibility to visceral leishmaniasis in Boxer dogs. Genetic markers can explain phenotypic variance in both pro- and anti-inflammatory cytokines and in cellular immune responses, including antigen presentation. Many gene segments are involved in canine visceral leishmaniasis phenotype, with Natural Resistance Associated Macrophage Protein 1 (NRAMP1) as the most studied. This was related to both protection and susceptibility. In comparison with murine and human genetic approaches, lack of knowledge in dogs is notorious, with many possibilities for new studies, revealing a wide field to be assessed on canine leishmaniasis susceptibility research.

1. Introduction

Leishmaniases are protozoan diseases caused by *Leishmania* spp. and transmitted by the bite of infected phlebotomine sand flies (Werneck, 2014). Visceral leishmaniasis is the most severe form of this disease (Brasil, 2006, 2009) which is caused by *Leishmania infantum* in Latin America and in the Mediterranean region (Ready, 2014; Werneck, 2014).

The domestic dog is the main reservoir of *L. infantum* (Ready, 2014), showing a diverse clinical spectrum, ranging from asymptomatic to highly symptomatic (Mancianti et al., 1988). The progression of *Leishmania* infection and its clinical manifestations result from complex interactions between the host immune response and the parasite (Maia and Campino, 2012; Brachelente et al., 2005; Santos-Gomes et al., 2002).

Susceptibility to canine leishmaniasis varies among individuals. Some dogs are able to control the infection by eliminating the parasite or by restricting the parasite burden, remaining consistently subclinical (Baneth et al., 2008). In this sense, dog breed seems to influence the pathological process, whether regarding susceptibility (Sanchez-Robert et al., 2005) or resistance (Solano-Gallego et al., 2000).

Upon immunological aspects, classically, Th1 pathway, when

Abbreviations: Nramp1, Natural Resistance Associated Macrophage Protein 1; PCR, polymerase chain reaction; CBD1, canine β-defensin-1; SNPs, single-nucleotide polymorphisms; ELISA, Enzyme Linked Immunosorbent Assay; TNF-α, tumor necrosis factor alpha; IL, interleukin; IFN-γ, interferon-gamma; TLR2a, Toll-like receptor 2 agonist; CanL, canine leishmaniasis; DTH, delayed-type hypersensitivity; LPA, lymphocyte proliferation assay; IgG, Immunoglobulin G; qPCR, quantitative PCR; FNA, fine needle aspiration; TGF-β, transforming growth factor beta; LPS, lipopolysaccharide; TLR, Toll-like receptor; NLR, NOD-like receptors; Th1, Type 1 T helper; Th2, Type 2 T helper; Th17, Type 17 T helper; Treg, regulatory T cell; IFI, indirect immunofluorescence; MHC, major histocompatibility complex; DLA, dog leukocyte antigen; GWAS, genome-wide association study; CMI, cell-mediated immunity; NF-κB, kappa light polypeptide gene enhancer; TLE1, transducin-like enhancer of split 1 gene; CFA, *Canis familiaris* chromosomes; Slc11a1, Solute carrier family 11 member a1; PRRs, prototype pattern-recognition receptors; PAMPs, pathogen-associated molecular patterns; DAMPs, danger-associated molecular patterns

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predominant, has been associated with protection against canine leishmaniasis (Gradoni, 2015; Barbiéri, 2006), whereas high no protective levels of antibodies have been showed in sick dogs (Barbiéri, 2006). However, a true multifactorial complex with several pathways, such as Th1, Th2, Treg, Th17 can be involved in leishmaniasis (Srivastava et al., 2016). This characteristic contributes to a battle between acute disease and the parasite elimination attempt (Cecílio et al., 2014; de Lima et al., 2007), and not always the clinical aspects reflect the parasite load (de Vasconcelos et al., 2016; Borja et al., 2016).

In relation to genetic approaches, differently from the human and murine models, which are extensively investigated regarding genetic susceptibility to both cutaneous and visceral leishmaniasis (Sophie et al., 2017; Hernández-Rivera et al., 2016; Fattahi-Dolatabadi et al., 2016; de Araujo et al., 2015; Hajilooi et al., 2015; Ortiz-Flores et al., 2015; Castellucci et al., 2014; Kumar et al., 2014; Ejghal et al., 2014; Fakiola et al., 2013; Sans-Fons et al., 2013; Sohrabi et al., 2013; Mehrotra et al., 2012; Rasouli et al., 2012; Fakiola et al., 2011; Frade et al., 2011; Castellucci et al., 2010; Farouk et al., 2010; Sakthianandeswaren et al., 2010; Depledge et al., 2009; Sakthianandeswaren et al., 2009; Alonso et al., 2007; Jamieson et al., 2007; Jeronimo et al., 2007; Havelková et al., 2006; El-Safi et al., 2006; White et al., 2005; Mohamed et al., 2004; Bucheton et al., 2003; Vladimirov et al., 2003; Badalová et al., 2002; Beebe et al., 1997), studies on this topic in dogs are scarce and restrict on its visceral form, probably due to the important epidemiological role that this host has in the severe anthropozoonotic visceral cycle (Ready, 2014; Brasil, 2006, 2009).

Therefore, the aims of this systematic review were to describe and discuss the scientific findings on genetic susceptibility to canine leishmaniasis, considering breed factors and immunological concepts, and determine the gaps found in this field of knowledge.

2. Methodology

2.1. Selection of studies and data extraction

Literature searches were conducted between April and June 2017 at two databases: PubMed and Google Scholar. The search strategy, including the index terms and the inclusion and exclusion criteria are presented in Chart 1. Additional papers were searched in the reference lists within the publications.

2.2. Evaluation of limitations and potential bias of the publications included

In order to evaluate the quality of publications, we observed 10 questions, based on Strobe and Strega Statements (Little et al., 2009), which are shown in Chart 2.

3. Results

3.1. Characteristics and main limitations of the studies included in this systematic review

At the first search, 1222 and 1795 articles were found in PubMed and Google Scholar databases, respectively. Among these 3017 titles or abstracts initially identified, only 12 publications that met the inclusion criteria were analyzed in detail, and then included in this review. They comprised breed immunological studies, genome wide association, or other studies on gene polymorphisms or gene sequencing, and transcription approaches. All papers were found in PubMed database, and neither Google Scholar nor the reference lists searched added a new article to this review.

The main characteristics of the studies are show in the Table 1.

In relation to the main limitations, two studies do not state if the association was the first report of a genetic association, a replication effort, or both. None of the selected studies clearly explains how study sample size was defined. As for characteristics of the study participants, none consider the nutritional condition or co-infections of the evaluated animals or cells donors, and eight papers do not give some other important characteristics of dogs regarding potential confounders, such as description and distribution of the purebreds involved, age of dogs, or period in the endemic area (exposure time). Eight papers do not address the infection dynamics and two showed this type of monitoring only for part of the evaluated dogs. For *Leishmania* diagnostic criteria, five papers that used molecular methods do not perform it from target tissues of *Leishmania* infection as lymph node or bone marrow, using blood as sample; one does not state the diagnostic method and the other does not clearly mention the tissue used in the molecular method; two studies show exclusively immunological diagnostic methods by serology and delayed-type hypersensitivity (DTH) test. Furthermore, six studies do not describe efforts to address potential bias and nine do not show multivariate control in addition to genetic factors; seven studies do not discuss important limitations such as source of potential bias, and four do not provide, or do it partially, necessary cautions with relation to interpretation of results.

In order to better evaluate the studies, a score rate was created according to the adequacy to each point of the Chart 2. Each totally full agreement received 1 point, those with partial agreement received 0.5 point, and totally disagreement received zero. The final score varies from zero to ten and categories not applicable received 1 point to not

Search strategies:
• Pub Med: (Leish*) and (dog or canine or canidae) and (mutations or polymorphisms, genetic or genetic or resistance, disease or susceptibility, disease or breed susceptibility or breed resistance or parasite load or healing, wound or immunology or clinical status) • Google Scholar: allintitle: (*Leishmania* OR leishmaniasis OR leishmaniosis) (dog OR Polymorphisms OR mutation OR Genetic OR resistance OR susceptibility OR "wound healing" OR immunology OR "parasite load" OR "clinical status")
Inclusion criteria:
Studies that present associations between canine genetic factors, including polymorphisms and breed variations, with: resistance or susceptibility to visceral leishmaniasis; immune response to *Leishmania* infection; parasitic load of *Leishmania* spp.; wound healing associated with leishmaniasis; clinical status in *Leishmania* infected dogs.
Exclusion criteria:
Studies on human, murine or other animal models; cutaneous leishmaniasis studies.

Chart 1. Search strategies and inclusion and exclusion criteria applied in the systematic review upon the genetic aspects related to canine visceral leishmaniasis.

1. Show whether the association is the first report of a genetic association, a replication effort, or both.
2. Explain clearly how study size is determined.
3. Provide some important characteristics of study participants and information on potential confounders.
4. Describe laboratory methods.
5. Show satisfactory *Leishmania* diagnostic criteria for inclusion criteria.
6. Describe efforts to address potential bias.
7. Show multivariate analysis model, in addition to genetic factors.
8. Summarize key results with reference to study objectives.
9. Discuss important limitations, such as source of potential bias.
10. Provide necessary cautions in relation to interpretation of results.

Chart 2. Conditions for assessment of the quality of the studies included in this systemic review on canine genetic susceptibility to visceral leishmaniasis.

impair the paper ratings.

The evaluation of studies is present in the Chart 3.

3.2. Summary of information

3.2.1. Breed-related studies on immunology

Martínez-Orellana et al. (2017) performed an experiment using three groups of dogs: healthy seronegative or low positive Ibizan Hounds; other purebred or mixed breed seropositive ill dogs; and healthy dogs (control). In result, only the Ibizan Hounds showed significant increase in Tumor necrosis factor alpha (TNF-α) production when whole blood stimulation with *L. infantum* antigen (LSA) + Toll-like receptor 2 agonist (TLR2a) - Pam3CSK4 was compared with TLR2a alone. These dogs also showed significantly higher levels of TNF-α after stimulation with LSA when compared with medium alone. Furthermore, significantly higher concentrations of TNF-α and Interleukin (IL) - 6 were detected in dogs of this breed, especially for the TLR2a and LSA + TLR2a treatments with the combination of LSA + TLR2a, promoting a synergistic pro-inflammatory effect with TNF-α. Additionally, after whole blood stimulation with LSA and Concanavalin A, significantly higher Interferon-gamma (IFN-γ) concentrations were detected in Ibizan Hounds compared with those of seropositive ill dogs. Interestingly, clinically healthy Ibizan Hound dogs presented statistically significant lower levels of antibodies than dogs with clinical leishmaniasis.

In order to test an immunological hypothesis related to Ibizan Hound dogs, Solano-Gallego et al. (2000) examined two populations: one composed of Ibizan Hounds and another of dogs of other breeds. The authors tested the presence of *Leishmania*-specific cellular immunity by using a delayed type hypersensitivity test (DTH) and for the presence of *Leishmania*-specific humoral immunity by using an Enzyme Linked Immunosorbent Assay (ELISA) test. All dogs were clinically healthy. Among the Ibizan Hounds, 81% were DTH positive whereas only 48% of the other dogs were positive for this test. Statistical association was found between Ibizan hounds and positive DTH response.

In the same study, it is worth highlighting that dogs from both populations that tested positive, Ibizan Hounds or not, did not differ regarding intensity of the DTH reaction as measured by reaction diameter in this research (Solano-Gallego et al., 2000). Therefore, the authors suggest that dogs of other breeds are as capable as the Ibizan Hounds of responding to infection, with the latter responding however more uniformly with a positive DTH response, being considered more resistant to *Leishmania* than other canine breeds. The study showed that Ibizan Hound dogs stand a significant cellular response to *Leishmania* infection, suggesting that this breed constitutes a special group of dogs regarding the immune response against *Leishmania* infection.

3.2.2. Studies on gene polymorphisms

In three mongrel dog populations from Brazilian and Italian endemic areas, da Silva et al. (2017) assessed the association between single-nucleotide polymorphisms (SNPs) in the canine β-defensin-1 (CBD1) gene and the infection by *L. infantum*. Nine polymorphic sites were detected, but only SNPs 3, 4, 7, and 8 were associated with parasite detection by real-time polymerase chain reaction (PCR), and only in dogs from southern Italy. The informative sites were found in the 5′ untranslated region (position in chromosome 16: 58881447) and in the intronic region (positions in chromosome 16: 58881356, 58881297, 58881294, 58881277, 58881159, 58881122, 58881093, 58881081). In addition, the genotypes A/A and T/T within the SNPs 3 and 8, respectively, were associated with risk of *L. infantum* infection, and the genotypes A/C and A/G within the SNPs 4 and 7, respectively, were associated with protection against *L. infantum* infection. No haplotypes were found to be associated with parasite detection in the studied dog populations. The results indicated that these SNPs could be potential genetic markers for the study of *L. infantum* infection susceptibility/resistance in dogs, and it was suggested that the CBD1 gene could be involved in the canine immune response to *L. Infantum* infection.

In a Genome-wide association study (GWAS), Batista et al. (2016) scanned 110,165 SNPs in order to identify chromosomal regions associated with DTH test, lymphocyte proliferation assay (LPA), and cytokine responses to further understanding of the role played by cell-mediated immunity (CMI) in the outcome of natural *Leishmania infantum* infection in dogs. Interestingly, DTH + /LPA + dogs showed increased IFN-γ and TNF-α levels and mild parasitism in lymph node, whereas DTH − /LPA + dogs, in spite of the increased IFN-γ, also showed increased IL-10 and TGF-β levels and the highest parasite load in lymph node. Genetic markers explained, respectively, 87%, 16%, 15%, and 11% of phenotypic variance in TNF-α, TGF-β, DTH, and IL-10. Lymphocyte proliferation was dependent on parasite load,

Table 1
Main characteristics of the studies included in this systematic review upon genetic aspects in canine visceral leishmaniasis susceptibility.

Author study/ year	Type of study	Case size sample	Control size sample	Main *Leishmania* diagnostic tools	Main immunological conclusions	Main genetic conclusions
da Silva et al. (2017)	Study on gene polymorphisms.	134 dogs (*Leishmania* positive dogs).	253 dogs (*Leishmania* negative dogs).	Real-time PCR from blood.	Suggestion that the CBD1 gene may be involved in the immune response of dogs to *L. Infantum* infection.	Nine polymorphic sites were detected, but only SNPs 3, 4, 7, and 8 were associated with *L. infantum*.
Martínez-Orellana et al. (2017)	Breed-related study on immunology.	17 clinically affected dogs from a low endemic area; 21 healthy seronegative or low positive Ibizan Hounds from a high endemic area.	11 healthy seronegative dogs.	ELISA; Cytology of lesions or histology or immunochemistry in some cases; PCR from blood.	Highest TNF-α, IL-6, and IFN-γ levels, in addition to lower antibodies levels in Ibizan hound; Pam3CSK4 (TLR2a) involved.	Suggestion of resistance to clinical CanL for Ibizan Hound dogs.
Batista et al. (2016)	Study on gene polymorphisms (Genome-wide association study).	100 dogs (positive in at least one of PCR, DHT test, or LPA).	17 dogs (negative for DHT test, LPA, parasitological, or anti-*L. infantum* IgG).	ELISA; DHT test; LPA; qPCR from lymph node FNA biopsies or buccal and conjunctival swabs.	DHT test + /LPA + dogs: increased IFN-γ and TNF-α levels and mild parasitism; DHT test − /LPA + dogs: increased IFN-γ, IL-10, TGF-β levels and the highest parasite load.	Genetic markers explained phenotypic variance in TNF-α, TGF-β, DHT test and IL-10.
Turchetti et al. (2015)	Gene transcription study.	Six macrophage samples resistant to *Leishmania* infection.	Six macrophage samples susceptible to *Leishmania* infection. Four positive controls treated with LPS.	ELISA and PCR from Blood.	Suggestion of involvement of TLRs against *Leishmania infantum* only by constitutive transcripts.	No interference of constitutive transcription of Nramp1, TLR, and NLR in intracellular survival of *Leishmania infantum*.
Utsunomiya et al. (2015)	Study on gene polymorphisms.	20 *Leishmania* positive dogs.	28 *Leishmania* negative dogs.	ELISA and PCR from blood.	Involvement of Th1 and macrophage modulation by interleukins 2, 15, IFN-γ, and Notch signaling.	Two candidate *loci* on autosomes 1 and 2 related to protection against *Leishmania infantum*.
Quilez et al. (2012)	Study on gene polymorphisms (Genome-wide association study) and genomic selection analysis.	104 CanL affected Boxer dogs.	115 *Leishmania* healthy infected Boxer dogs (absence of CanL with evidence of previous infection).	qPCR from blood and occasionally from other tissues and ELISA. Direct parasite detection and anti-*Leishmania* immune tests for most animals.	Not shown directly, but inferred host response based on human and mouse knowledge employed.	Many gene segments are involved in CanL phenotype. Genetic variance may explain 60% of the phenotypic variance.
Bueno et al. (2009)	Gene sequencing and expression study.	Two macrophage samples resistant to *Leishmania* infection for cloning and sequencing analysis. 10 serological positive dogs (five symptomatic and five asymptomatic) and other 10 (five symptomatic and five phenotypically resistant with at least 6 months asymptomatic) for splenic expression analysis.	Two macrophage samples susceptible to *Leishmania* infection for cloning and sequencing analysis. Five serological negative dogs for splenic expression analysis.	IFI and ELISA.	No significant differences in the levels of IFN-γ, IL-4, and IL-12 between symptomatic and asymptomatic dogs, neither between resistant and susceptible dogs.	Absence of polymorphism linked to resistance or susceptibility to CanL.
Sanchez-Robert et al. (2008)	Study on gene polymorphisms.	164 severely ill dogs.	99 healthy dogs living in an endemic area that could have been in contact with the parasite at some time.	PCR and serology.	Suggestion that CanL susceptibility is associated with variation in the immune functions of NRAMP1 gene.	Two polymorphisms associated with increased risk of CanL.
Sanchez-Robert et al. (2005)	Study on gene polymorphisms.	40 CanL dogs.	57 healthy dogs living in an endemic area that could have been in contact with the parasite at some time.	Not mentioned.	Suggestion that CanL protection or susceptibility are associated with variation in the immune functions of NRAMP1 gene.	Alleles associated with protection or susceptibility to CanL in Boxer dogs.
Quinnell et al. (2003)	Gene sequencing study.	86 dogs with parasitological or immunological evidence of *Leishmania* infection.	40 *Leishmania* negative dogs.	PCR of bone marrow, ELISA, and LPA.	Suggestion of susceptibility to CanL related to MHC class II.	Allele DLA-DRB1*01502 significantly associated with levels of anti-*Leishmania* IgG and parasite status.

(*continued on next page*)

Table 1 (*continued*)

Author study/ year	Type of study	Case size sample	Control size sample	Main *Leishmania* diagnostic tools	Main immunological conclusions	Main genetic conclusions
Altet et al. (2002)	Gene sequencing study.	84 susceptible dogs with positive *Leishmania* PCR result.	33 clinically resistant dogs with positive DTH test.	PCR of bone marrow and DTH test.	Suggestion of association between Nramp1 and susceptibility to CanL.	Genetic variance in Nramp1 could be associated with possible functional mutations related to susceptible or protective immune responses.
		Two susceptible Beagles experimentally infected (predominant cellular immune response).	Two resistant Beagles experimentally infected (predominant humoral immune response).			
Solano-Gallego et al. (2000)	Breed related study in immunology.	31 Ibizian Hounds.	25 dogs of other breeds.	DTH test and ELISA.	Statistical association between Ibizian Hounds and positive DTH response.	Suggestion of genetic clinical resistance background for Ibizan Hound dogs.

PCR: polymerase chain reaction; CBD1: canine β-defensin-1; SNPs: single-nucleotide polymorphisms; ELISA: Enzyme Linked Immunosorbent Assay; TNF-α: Tumor necrosis factor alpha; IL: Interleukin; IFN-γ: Interferon-gamma; TLR2a: Toll-like receptor 2 agonist; CanL: canine leishmaniasis; DTH: delayed-type hypersensitivity; LPA: lymphocyte proliferation assay; IgG: Immunoglobulin G; qPCR: quantitative PCR; FNA: fine needle aspiration; TGF-β: Transforming growth factor beta; LPS: Lipopolysaccharide; TLR: Toll-like receptor; Nramp1: Natural Resistance Associated Macrophage Protein 1; NLR: NOD-like receptors; Th1: Type 1 T helper; IFI: indirect immunofluorescence; MHC: major histocompatibility complex; DLA: dog leukocyte antigen.

suggesting that *L. infantum* was not able to induce an effective T cell response under low parasite load. Additionally, genetic analyses indicated no genetic control underlying LPA response or low statistical power. It is worth highlighting that the statistical analyses showed no association between clinical and CMI profiles and that even DTH + dogs exhibiting a predominantly Th1 response showed clinical signs of canine leishmaniasis (CanL).

In the same GWAS, the authors showed that regions associated with TNF-α include the genes IL12RB1, JAK3, CCRL2, CCR2, CCR3, CXCR6 involved in cytokine and chemokine signaling; other TNF-α related genes are IFI30 and XCR1, in addition to other positional candidate genes in the vicinity of less significant markers such as ITGA4, FCRL1, FCRL4, CD5L, and TP53BP2. Regions associated with LST contain COMMD5 and SHARPIN involved in the regulation of nuclear factor of kappa light polypeptide gene enhancer (NF-κB) signaling. The region associated with IL-10 includes LTBP1, which is involved in the activation of TGF-β, and RASGRP3, which is implicated in the down-regulation of proinflammatory cytokines. Both LTBP1 and RASGRP3 are involved in T regulatory lymphocytes differentiation, pointing to the likely induction of TGF-β activation and its effect on Treg differentiation that would favor the release of IL-10. As for TGF-β, candidate genes included CCR8, CX3CR1, TNFSF4, TNFSF18, SELE, SELL, and SELP. The results indicated that a baseline TGF-β response is ensured by many genetic variants. With respect to IFN-γ, despite the two significant markers, the phenotypic variance explained by the markers was 0%, suggesting no genetic control underlying IFN-γ response of peripheral blood mononuclear cells in LPA or low statistical power to detect significance (Batista et al., 2016).

Using 149,648 SNP markers genotyped in mixed-breed dogs from a high endemic area in Brazil, Utsunomiya et al. (2015) found two candidate *loci* on canine autosomes 1 and 2. The positional association on chromosome 2 was related to a predicted DNAse sensitive site in CD14 + monocytes that serve as a cis-regulatory element for the expression of interleukin alpha receptors 2 (IL2RA) and 15 (IL15RA) in macrophages. The associated marker on chromosome 1 was located between two predicted transcription factor binding sites, regulating the expression of the transducin-like enhancer of split 1 gene (TLE1). This gene is also known as Q6JDG1_CANFA and participates in the Notch signaling pathway, which is critical for macrophage activity and CD4 + T cell differentiation. The authors highlight that this is a preliminary study and that the fine mapping of these candidate genes using re-sequencing data may contribute to the identification of variants implicated in susceptibility to visceral leishmaniasis in dogs.

Applying GWAS and genomic selection, Quilez et al. (2012) sought to identify individual disease *loci*, quantify the genetic component of phenotypic variance, and test whether genome-wide SNP data could predict the clinical progression of leishmaniasis in infected dogs. To this end, they conducted a case-control study with a population of 219 Boxer dogs (115 healthy infected and 104 affected dogs) that were genotyped for ~170,000 SNPs. All dogs had genotypes for 126,607 SNPs distributed across the genome. Three statistical models were applied to correct confounding effects. When no covariates were included, the data showed the strongest associations on *Canis familiaris* chromosomes (CFA) 1, 4, and 20, although none of these were statistically significant at a genome-wide level. The association remained on CFA 1 and 4 when confounding effects were accounted, but it was also not significant at genome-wide level. The authors examined candidate *loci* previously associated with susceptibility to leishmaniasis in mice and men, and successfully mapped 78 of them to their orthologues in the dog genome. The analyses resulted in 4751 SNPs in 37 sets that were tested one at a time for association with phenotype. Sets on CFA 4 spanned the region 61.2–76.9 MB and genomic selection estimated markers in this region to have the greatest effect on the phenotype. In conclusion, their results suggest that many different gene segments contribute to the phenotype in *Leishmania* infection in dogs and genetic variance may explain as much as 60% of the total phenotypic variance.

Author Study/ Year	Criterion number for assessment of the quality of the studies										
	1	2	3	4	5	6	7	8	9	10	Final score
da Silva et al. (2017)	++	--	--	++	+-	--	--	++	++	++	5.5
Martínez-Orellana et al. (2017)	NA	--	--	++	+-	--	--	++	--	++	4.5
Batista et al. (2016)	++	--	--	++	++	++	++	++	--	--	6.0
Turcheti et al. (2015)	NA	--	+-	++	+-	--	--	++	++	++	6.0
Utsunomiya et al. (2015)	--	--	--	++	+-	++	--	++	--	++	4.5
Quilez et al. (2012)	++	--	+-	++	+-	++	++	++	++	++	8.0
Bueno et al. (2009)	--	--	--	++	+-	--	--	++	--	+-	3.0
Sanchez-Robert et al. (2008)	++	--	--	++	+-	++	--	++	--	++	5.5
Sanchez-Robert et al. (2005)	++	--	+-	+-	--	++	--	++	--	+-	4.5
Quinnell et al. (2003)	++	--	+-	++	++	++	++	++	++	++	8.5
Altet et al. (2002)	++	--	--	++	++	--	--	++	++	++	6.0
Solano-Gallego et al. (2000)	++	--	--	++	+-	--	--	++	--	--	3.5

Legend: 1-10: criterion number for assessment of the quality of the studies previously described in the methodology section; article adequately complied with the condition (two positive symbols), article did not refer to the procedure condition (two negative symbols), and article referred to the procedure but did not fully comply with the condition (a negative and a positive symbol); NA: not applicable. The final score varies from zero to 10, with two negative symbols on the chart adding zero point to the score, a negative and a positive symbol adding 0.5, and two positive symbols 1 point. NA was considered 1 point.

Chart 3. Assessment of the adequacy of the studies included in the systematic review on canine genetic susceptibility to visceral leishmaniasis.
Legend: 1–10: criterion number for assessment of the quality of the studies previously described in the Methodology section; article adequately complied with the condition (two positive symbols), article did not refer to the procedure condition (two negative symbols), and article referred to the procedure but did not fully comply with the condition (a negative and a positive symbol); NA: not applicable. The final score varies from zero to 10, with two negative symbols on the chart adding zero point to the score, a negative and a positive symbol adding 0.5, and two positive symbols 1 point. NA was considered 1 point.

In a case-control study conducted with 97 dogs of 14 different breeds, Sanchez-Robert et al. (2005) analyzed five polymorphisms of the Natural Resistance Associated Macrophage Protein 1 (Nramp1) gene. Forty of them were diagnosed with canine leishmaniasis and 57 were healthy dogs from the same breeds. To assess the role of Nramp1 gene in susceptibility to *Leishmania* infection in dogs, the promoter region was analyzed for polymorphisms and the microsatellite located in the intron 1 was tested. The sequencing analyses resulted in three new SNPs that gave rise to three new haplotypes (TAA, TGA, CGA). These three SNPs affect different genomic binding sites of eukaryotic transcription factors, which could suggest a possible role of differential gene expression. The sequencing also showed a variation from seven to nine in the number of Gs in the G-stretch, with G8 as the most frequent allele. Analysis of the intron 1 showed five different alleles (133, 137, 139, 141, 145), with alleles 141 and 145 as the most frequent. Haplotype frequency distributions showed significant differences between case and control populations, most likely due to the SNPs of the promoter region that were associated with case dogs. No significant differences were observed either for the G-stretch variation or the microsatellite.

Additionally, in the same study, the most frequent haplotypes included TAG-8-141, which was present in all of the breeds, in both case and control animals, and TAG-9-145, which was overrepresented in the control population and mostly found in Boxer dogs, suggesting that haplotype distribution is breed specific. TAG-8-141 allele was associated with susceptibility in Boxer dogs (which was not observed for the other breeds), whereas TAG-9-145 allele would be protective in homozygous state for the same breed. These results emphasize the potential of breed genetic background in canine leishmaniasis susceptibility.

Continuing the study on the role of Nramp1 gene in canine leishmaniasis, Sanchez-Robert et al. (2008) sequenced 7110 bp from the whole gene in 40 dogs from 11 different breeds, revealing 19 new polymorphisms. Two SNPs were located in the coding region (exons 8 and 12) and represent silent mutations. Fifteen SNPs and a 14 bp deletion were identified in intronic regions and one SNP in the 3′UTR. These new polymorphisms found, as the three SNPs previously published (Sanchez-Robert et al., 2005), the G-stretch and the microsatellite in intron 1 were analyzed in a case-control cohort including 164 dogs of different breeds and in an Ibizan Hound cohort with 58 dogs. The results showed that two of the 24 analyzed SNPs in canine Nramp1 (in intron 6: A4549G and one silent SNP in exon 8: C4859T) were significantly associated with increased risk for canine leishmaniasis. Interestingly, in the Ibizan Hound cohort no dog was affected by CanL, and six Nramp1 haplotypes appear to be specific to this breed.

3.2.3. Gene transcription and/or other sequencing studies

In an evaluation of basal transcription of genes associated with innate immunity in canine monocyte-derived macrophages from *Leishmania*-free dogs, Turchetti et al. (2015) did not find interference with constitutive transcription of Nramp1, TLR, and NLR in intracellular survival of *L. infantum*. Despite this, high levels of

constitutive expression of Nramp1 and TLR 2, 3, 4, 5, and 6 were found, suggesting that they may play a role in the recognition and control of pathogens by canine macrophages. In contrast, NOD2 and TLR9 expressions were negligible and likely to have secondary roles in macrophage-mediated innate immunity of dogs. However, significant negative correlation was found between NOD2 gene expression and intracellular survival of *L. infantum* in resistant macrophages, and it may be a relevant target for future investigations.

In a similar study, Bueno et al. (2009) compared the cDNA sequence and the expression of Nramp1 from 29 seronegative dogs whose macrophages were resistant or susceptible to *L. infantum* infection. Only four of these dogs were selected for cDNA sequencing, which showed no difference between resistant and susceptible animals. Moreover, expression of Nramp1 and cytokines (IFN-γ, IL-4, and IL-12) was measured in the spleen of 15 animals, including five seropositive symptomatic dogs, five seropositive asymptomatic dogs, and five seronegative dogs. Ten other infected dogs were included after six months of monitoring and classified as resistant or susceptible according to clinical evolution. There was no significant difference between the levels of mRNA for Nramp1, IFN-γ, IL-4, or IL-12 between symptomatic and asymptomatic dogs, although the Nramp1 mRNA levels tended to be higher in asymptomatic dogs.

In a cohort of Brazilian mongrel dogs exposed to natural *L. infantum* infection, Quinnell et al. (2003) examined the relationship between dog leukocyte antigen (DLA) class II alleles (DRB1, DQA1, DQB1) and the course of infection, assessed by clinical status, parasitology, and specific immune responses. DLA-DRB1 genotype was significantly associated with levels of anti-*Leishmania* Immunoglobulin G (IgG) and parasite status assessed by PCR. DLA-DRB1*01502 was the only allele significantly associated and dogs with this allele showed higher levels of specific IgG and increased risk for being parasite positive. No significant associations were observed for DLA-DQA1 or DLA-DQB1 alleles. These results suggest that the DLA-DRB1 *locus*, which is related to major histocompatibility complex (MHC) class II in dog, plays a role in determining susceptibility to CanL.

In another study, Altet et al. (2002) sequenced and mapped the canine Nramp1 gene aiming to identify mutations that could be associated with resistance or susceptibility to *Leishmania* infection. A case-control study was performed with 33 healthy dogs that showed positivity in *Leishmania* DTH and were classified as resistant and 84 symptomatic dogs that tested positive in PCR of bone marrow and were considered susceptible. Significant differences between resistant and susceptible dogs were observed in the frequency of allele 145 of the intron 1 microsatellite, which was only found in a homozygous state in the susceptible group, except for one resistant dog. Additionally, four unrelated Beagle dogs experimentally infected with *L. infantum* were used in search for mutations with functional implications. Two of them were considered resistant and the other two were considered susceptible to leishmaniasis according to immune response. The authors found two important mutations in susceptible dogs: a G-rich region in the promoter and a complete deletion of exon 11, which encodes the consensus motif of the protein. Therefore, the results suggested an association between NRAMP1 and leishmaniasis, indicating a role of this gene in disease susceptibility.

3.3. Gaps revealed in this field of knowledge

Among the gaps, it is notorious that usually it is not established a differentiation between clinical health dogs and infected ones in the studies, as well as the papers no consider the possibility of this host be a source of infection even without showing clinical signs; there is a scarcity of immunological and genetic analyzes by canine breed; there are few studies upon directly genetic aspects related to canine susceptibility to leishmaniasis and therefore, the genetic markers studied sometimes are few or are verified in few approaches or in small samples; the better analysis in this sense was the GWAS, but they were only two studies. In this way, although significant results, the canine analyzes are so far, in number of genetic studies, from human and murine approaches.

4. Discussion

Most of the reviewed papers directly show or suggest immunological aspects of the susceptibility to CanL. In fact, the immune response mounted by dogs against *Leishmania* appears to be closely related to progress from a subclinical state into clinical illness and are related to disease resistance (Barbiéri, 2006). In this sense, the breed immunological studies point to particularities of hosts in the response against *Leishmania* and suggest a genetic background in Ibizan Hound dogs. The significant increase of TNF-α and IFN-γ reported by Martínez-Orellana et al. (2017) after stimulation techniques in dogs of this breed points to a Th1 pathway which, when predominant, is associated with protection against canine leishmaniasis (Gradoni, 2015; Barbiéri, 2006). Furthermore, the lower level of antibodies detected in Ibizan Hounds compared with that of clinically ill dogs are in agreement with the scientific literature, considering that high anti-*Leishmania* antibody titers are not protective in CanL and are associated with symptomatic dogs (Barbiéri, 2006; Pinelli et al., 1994), as well as with positive splenic culture (dos-Santos et al., 2008).

However, the Ibizan Hounds included in the study conducted by Martínez-Orellana et al. (2017) were clinically healthy but some of them were low seropositive. In this context, there should be a differentiation between clinical resistance and susceptibility to infection, considering that some reports have previously shown the absence of association between clinical aspects and parasite burden (de Vasconcelos et al., 2016; Borja et al., 2016), with asymptomatic dogs being a source of infection to sand fly vectors (Laurenti et al., 2013; Alvar et al., 2004). Also in this sense, the IL-6 level found after Ibizan Hound whole blood stimulation may point to active leishmaniasis, because this cytokine is suggested as a marker of active disease in the canine host (de Vasconcelos et al., 2016; de Lima et al., 2007). Therefore, it would be interesting to be conduct parasite load and xenodiagnostic approaches in dogs of this breed in *Leishmania* endemic areas.

Also in this sense, the not affected cohort of Ibizan Hounds dogs reported by Sanchez-Robert et al. (2008) and the significant association between Ibizan Hounds and positive DTH response (Solano-Gallego et al., 2000) are in agreement with the clinical resistance hypothesis. In relation to DTH, in fact, symptomatic dogs have been associated with T cell immunological changes such as absence of DTH to *Leishmania* antigens (dos-Santos et al., 2008; Barbiéri, 2006; Pinelli et al., 1994). However, it is important to note that dogs of other breeds also responded to DTH, although less uniformly (Solano-Gallego et al., 2000). Furthermore, there are conflicting results in mongrel dogs, presenting high frequency of asymptomatic dogs, negative DTH, and positive specific IgG antibody response (Silveira et al., 2012); as well as in purebred dogs, showing DTH + animals not protected against clinical manifestations (Batista et al., 2016). In addition, the alleles related to susceptibility and protection in Boxer dogs (Sanchez-Robert et al., 2005) indicate other possible variations associated with breed genetic background, and the simple report of *Leishmania* detection in different breeds may highlight possibilities of susceptibility, as previously related for Boxers and Cockers (França-Silva et al., 2003) or Fox Hounds (Gaskin et al., 2002). Therefore, it would be interesting to conduct large-scale immunological analyses according to breed so that possible breed particularities could be verified.

Other interesting aspects to be considered include an epidemiological analysis of breeds as risk factor in endemic areas or genetic origin analysis related to the breed mixture in mongrel dogs and the achievement of association tests between the origin results and immunological aspects, as it occurs in human analysis using genomic ancestry evaluation (Furini et al., 2016; Cassiano et al., 2015).

However, in dogs, the high number of existing breeds (Serpell and Duffy, 2014) is an important complicating factor, which would require large and expensive approaches.

Regarding the suggestion of CBD1 gene involvement in canine immune response to *L. infantum* infection (da Silva et al., 2017), it is important to highlight that polymorphisms in this gene have already been associated with respiratory and cutaneous diseases in dogs (Erles and Brownlie, 2010; Van Damme et al., 2009), with detection of related transcriptions in skin (Erles and Brownlie, 2010). In fact, defensins are antimicrobial peptides expressed in a variety of epithelial cells and sometimes in leukocytes that play a role in the innate immune system due to their antimicrobial, chemotactic and regulatory activities (Hazlett and Wu, 2011). It is worth noting that, in relation to chemotactic activities, defensins can enhance the adaptive immune response by chemoattracting immature DCs (iDCs), and it has been demonstrated that β-defensins might function as the most potentially important chemoattractants of iDCs (Hazlett and Wu, 2011). In this context, it is known that macrophages and dendritic cells play a key role in initiating, developing, and maintaining protective immunity against *Leishmania* (Srivastava et al., 2016). Nevertheless, at the same time, there is evidence supporting a dichotomous role of dendritic cells in cutaneous leishmaniasis to modulate immune response for both resistance and susceptibility (Feijó et al., 2016). Therefore, the SNPs in CBD1 gene reported by da Silva et al. (2017) may be potential genetic markers for the study of *L. infantum* infection susceptibility/resistance in dogs.

Regarding the immunological phenotypes and their genetic markers demonstrated by Batista et al. (2016), despite the absence of association between clinical manifestations and cell-mediated immunity, increased TNF-α and DHT test positive reaction associated with lower parasitism points to a relative resistance that can be related to the Th1 pathway (Gradoni, 2015; Barbiéri, 2006), considering that the DHT test indicates T-cell function (Pinelli et al., 1994). In addition, the regions associated with the DHT test were related to NF-κB signaling, which is a proinflammatory signaling pathway involved in multiple processes, including expression of proinflammatory cytokine genes such as IL-1 and TNF-α, chemokines, and adhesion molecules (Lawrence, 2009). In contrast, the phenotypic variance of TGF-β and IL-10 is associated with regulatory pathway, which could, in fact, contributes to failure of pathogen elimination and disease progression (Trinchieri, 2007). Moreover, their relation with negative DHT test reaction and higher parasite load are in agreement with this idea.

With respect to the positional association demonstrated by Utsunomiya et al. (2015) on chromosome 2, this was related to IL2RA and IL15RA, which are receptors with gene expression controlled by IFN-γ - the main cytokine associated with Macrophage 1 (M1) activation and the main Th1 cell product (Martinez and Gordon, 2014). Furthermore, in supernatants of stimulated peripheral mononuclear cells from asymptomatic dogs, significantly higher level of IL-2 and tumor necrosis factor were found in comparison with those from symptomatic and control uninfected dogs (Pinelli et al., 1994). Concerning IL-15, this cytokine as well as IFN-γ induce the killing of *Leishmania infantum* in macrophages by inducing IL-12 production (D'Agostino et al., 2004). In relation to the Q6JDG1_CANFA gene and Notch signaling, this pathway is necessary for IFN-γ secretion by T Helper 1 cells during infection with *Leishmania major*, being both Notch1 and Notch2 signaling expressed on activated CD4 + T cells, redundant in driving Th1 differentiation (Auderset et al., 2012).

Concerning NRAMP1, this was the most studied gene associated with canine leishmaniasis susceptibility (Turchetti et al., 2015; Bueno et al., 2009; Sanchez-Robert et al., 2008; Sanchez-Robert et al., 2005; Altet et al., 2002). NRAMP1 is a former but more familiar designation to Solute carrier family 11 member a1 (Slc11a1), which is a proton/divalent cation antiporter. In fact, it was originally described regulating resistance and susceptibility to *Salmonella typhimurium*, *Leishmania donovani*, and *Mycobacterium bovis* in mice (Blackwell et al., 2003). It has many effects on macrophage activation, such as regulation of chemokines, IL-1b, inducible nitric oxide synthase, major histocompatibility complex class II molecules, TNF-α and nitric oxide release (Blackwell et al., 2001), and has been related to both cutaneous and visceral leishmaniasis in humans (Hernández-Rivera et al., 2016; Ortiz-Flores et al., 2015; Ejghal et al., 2014; Castellucci et al., 2010; El-Safi et al., 2006; Mohamed et al., 2004; Bucheton et al., 2003).

In this sense, the genetic alterations found in canine NRAMP1 by Altet et al. (2002) suggest the generation of a functional impairment towards visceral leishmaniasis susceptibility in dogs, and the results of Sanchez-Robert et al. (2005, 2008), are also in agreement with the susceptibility or resistance idea related to variations of this gene. Whereas Turchetti et al. (2015) only highlight the possible roles of NRAMP1 in the recognition and control of pathogens by canine macrophages, due to its high levels of constitutive transcripts detected, Bueno et al. (2009) found no difference in the cDNA sequence of NRAMP1 between resistant and susceptible canine macrophages, neither in its splenic transcripts between symptomatic and asymptomatic dogs.

As for the report of TLR, in fact, these are prototype pattern-recognition receptors (PRRs) that recognize Pathogen-associated molecular patterns (PAMPs) from microorganisms or danger-associated molecular patterns (DAMPs) (O'Neill et al., 2013). They are located either on the plasma membrane or internal membranes of macrophages, DCs, NK cells, T and B lymphocytes, and after recognition of specific pathogen antigens, TLRs trigger NF-kB, which then proceeds to the nucleus and promotes the transcription and further synthesis of proinflammatory cytokines (Tuon et al., 2008). In this sense, regarding the TLR2 and TLR4 higher levels detected by Turchetti et al. (2015), in fact, TLR2 recognizes a wide range of PAMPs derived from various pathogens, including parasites, and both TLR2 and TLR4 are also implicated in the recognition of endogenous molecules that trigger the production of TNF-α, IL-12, and nitric oxide by macrophages (Kawai and Akira, 2009). TLR4 was also related to parasite control, probably due to the activity of iNOS in mice infected with *Leishmania major* (Kropf et al., 2004). In relation to TLR3, it can contribute to the recognition of *Leishmania donovani*, showing a role in the leishmanicidal activity of the IFN-γ-primed murine macrophages (Flandin et al., 2006); this receptor is located in intracellular endosomal membranes, recognizing double-stranded RNA and triggering NF-κB and the production of IFN (Tuon et al., 2008). The role of TLR5 in immunity against *Leishmania* infection is still unknown (Gurung and Kanneganti, 2015), whereas TLR6 and TLR1 are anchored to TLR2 and use the same signaling pathway (Tuon et al., 2008). Moreover, a diacylated lipopeptide ligand of TLR6 plays a host-protective role against experimental *Leishmania major* infection in mice (Pandey et al., 2014).

In spite of the negligible expression of TLR9 demonstrated by Turchetti et al. (2015) in canine macrophages, there is evidence that this TLR, myeloid dendritic cells, and IL-12 are functionally linked to the activation of Natural Killer cells in visceral leishmaniasis in the mouse model (Gurung and Kanneganti, 2015; Schleicher et al., 2007). However, *Leishmania* can utilize host proteins to regulate TLR signaling and LPG signaling, with TLR2 inhibiting TLR9 expression and anti-leishmanial responses in *Leishmania major* infected mice (Gurung and Kanneganti, 2015; Srivastava et al., 2013), which is a possible explanation for the TRL9 negligible expression. As for NOD2, it belongs to a NLR large family of intracellular PRRs and recognizes intracellular bacterial cell products. In relation to the possibility of NOD2 as a relevant target for research, in fact, its signaling leads to NF-κB and MAPK activation and induction of inflammatory cytokines and other anti-microbial genes, contributing to host defense (Kawai and Akira, 2009). Interestingly, Nascimento et al. (2016) showed that the NOD2-RIP2 pathway is activated in murine and human visceral leishmaniasis and plays a role in shaping adaptive immunity towards the Th1 profile.

With respect to the DLA-DRB1, Fakiola et al. (2013) established that common polymorphisms in the human leukocyte antigen (HLA)-

DRB1–HLA-DQA1 segment of the MHC region are genetic risk factors for visceral leishmaniasis. Their effects seem to cross the epidemiological divides of geography and parasite species, with analyses conducted in two different human populations in India and Brazil. In fact, these gene regions are related to MHC II and, although both MHC classes are related to parasite elimination, only MHC II is involved in total parasite clearance (Cecílio et al., 2014), being a good target to studies on polymorphisms to evaluate visceral leishmaniosis susceptibility in both dog in human researches.

Quilez et al. (2012), analyzing candidate genes and *loci* described in humans and mice (Bucheton et al., 2007; Jamieson et al., 2007; Jeronimo et al., 2007; Miller et al., 2007; Salih et al., 2007; Havelková et al., 2006; Baguet et al., 2004; Elso et al., 2004; Bucheton et al., 2003; Mohamed et al., 2003; Vladimirov et al., 2003; Badalová et al., 2002; Karplus et al., 2002; Peacock et al., 2002; Meddeb-Garnaoui et al., 2001; Beebe et al., 1997; Roberts et al., 1997; Cabrera et al., 1995), found some associations in dogs and highlighted different regions that could be explored in new approaches. In fact, a simple comparison between the number of human or murine researches cited throughout this review aiming to introduce or discuss the subject, and the only 12 canine articles directly included, (Martínez-Orellana et al., 2017; Batista et al., 2016; Turchetti et al., 2015; Utsunomiya et al., 2015; Quilez et al., 2012; Bueno et al., 2009; Sanchez-Robert et al., 2008; Sanchez-Robert et al., 2005; Quinnell et al., 2003; Altet et al., 2002; Solano-Gallego et al., 2000), indicate a sub-exploration of the canine genetic topic and reveal a wide field to be assessed on canine leishmaniasis susceptibility research.

Ultimately, in relation to limitations described in analyzed studies, they could have been mitigated with better study designs that considered a good definition of sample size, the nutritional data of dogs, and the inclusion of serological screen analyzes to considered co-infections, besides breed group formation when applicable. However, other characteristics related to infection dynamics and exposition time are difficult to be accessed in natural conditions and influence the clinical, immunological and parasitological parameters. Therefore in this case, experimental and controlled approaches in the canine model are usually more suitable to solve this kind of bias (Costa et al., 2013). Finally, it's suggested that diagnostic tools applied to visceral leishmaniasis include parasitological gold methodologies (De Vries et al., 2015) or molecular approaches using as samples target organs such as bone marrow, spleen and lymph node (Furtado et al., 2015), in complementation to serological analyses. Nevertheless, although the limitations found on the papers, the significant results presented in each one of them highlight their importance in this scarce field of knowledge.

5. Conclusions

Overall, the data reviewed in this study suggest a genetic clinical resistance background for Ibizan Hound dogs and that alleles could be associated with protection or susceptibility to visceral leishmaniasis in Boxer dogs.

Genetic markers can explain phenotypic variance in both pro- and anti-inflammatory cytokines and cellular immune responses, including antigen presentation.

Many gene segments are involved in CanL phenotype, with NRAMP1 as the most studied gene in canine leishmaniasis with data related both to protection and susceptibility, according to the variations found.

In a last analysis, based on the comparison between murine and human genetic approaches and the scarce number of published studies with dogs, we conclude that there is a notorious lack of research on this theme, with many possibilities for new studies in a sub-exploration and wide scientific area that needs to be more assessed on canine leishmaniasis susceptibility.

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Declaration of interest

None.

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