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PHENOTYPIC CHARACTERIZATION OF *RHIPICEPHALUS* SPP. RELATED TO BITES IN HUMANS IN SPAIN

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Abstract This study aimed to phenotypically characterize ticks from human bites in La Rioja and canine bites in the province of Valencia, Spain. Phenotyping was performed using a computerized image analysis system (CIAS, Computer picture Analysis System). The analysis by means of principal components of the morphological markers of ticks of the genus *Rhipicephalus* spp. of human and canine patients allows its separation into two groups with independent phenotypic characteristics. In the case of females, the principal component 1 shows 83.2% of the variance, explained by the length of the idiosoma and width of the scutum, while, in males, the principal component 1 represents 81.1% of the variance, explained by the length of the idiosoma and the length of the scutum.

Key words: *Rhipicephalus*, morphometry, phenotypic characterization, human, dog

INTRODUCTION

Diseases transmitted by vectors, according to the World Health Organization (WHO), represent more than 17% of infectious diseases, which cause more than one million deaths per year (WHO, 2016). Among the main vectors that cause these infectious diseases are the ticks of the Ixodidae family that transmit pathogens such as protozoa, viruses, bacteria and fungi, which affect humans with epidemic potential such as: *Borrelia burgdorferi sensu lato* (*sl)* (Lyme disease), *Anaplasma phagocytophilum* (human granulocytic anaplasmosis), encephalitis virus (tick-borne encephalitis) and Crimean-Congo hemorrhagic fever virus (Crimean-Congo hemorrhagic fever) (Estrada-Peña, 2015). In Spain, in recent years, there have been outbreaks of diseases transmitted by ticks to humans, among which the Crimean-Congo hemorrhagic fever virus (CCHF) has a greater global spread and a fatality rate of 10-40 % (De La Fuente et al., 2004; Márquez et al., 2005). This virus has been detected since 2010 circulating in ticks captured in the province of Cáceres, and in 2016 two cases have been diagnosed in humans. The first case was associated with contact with a tick in the province of Ávila and the second case was infection after close contact with the previous case in Madrid (Palomar et al., 2017).

The importance of the phenotypic study of ticks, from a health point of view, mainly lies in a correct identification, since certain diseases are transmitted exclusively by a tick species. The high specificity between vector and pathogen justifies the importance of morphological identification of the tick. The knowledge of their presence is decisive to establish measures of prevention and control in an area, region or country (Estrada-Peña and Jongejan, 1999; Barandika et al., 2010).

The present study aimed to phenotypically characterize Ixodidae related to bites in humans and canines in Spain, through traditional morphometry and geometric morphology.

MATERIALS AND METHODS

Study area. Ticks that were removed from humans and canines were analyzed in the study. Human ticks came from 2 municipalities (Logroño and San Román de Cameros) in the La Rioja province of Spain, and canine ticks came from the Paterna municipality in the Valencia province of Spain.

Parasitological material. Adult specimens (males and females) were analyzed in the study. The samples were preserved in 70% alcohol at room temperature. The number of adult specimens used for phenotyping is detailed in Table 1 and Table 2.

Taxonomic identification. Specimens were identified using a Nikon SMZ-U Zoom stereomicroscope (Nikon Corp., Tokyo, Japan) and taxonomic keys (Walker et al ., 2003). Taxonomic identification was confirmed by molecular techniques.

Morphometric methods. To perform the morphometric analysis of the adult ticks, a computerized image analysis system (CIAS, Computer picture Analysis System) was used. To obtain the digital images, a Nikon SMZ-U Zoom stereomicroscope, equipped with a digital camera, was used; Image capture and camera control were carried out with the KAPPA ImageBase Control program (KAPPA optoelectronics Inc., USA). For the measurements of various dimensions on the image of the specimens, the program Image - Pro ®Plus version 5.1 for Windows (Media Cybernetics Inc., Silver Spring, USA) was used. For their measurement the ticks were placed in a petri dish with paraffin. The measurements were taken according to Coimbra-Dores et al. (2016) and Sanchez et al. (2016). The morphometric parameters analyzed in adults of *Rhipicephalus* spp. are as follows (figure 1): idiosoma length (LI), idiosoma width (AI), scutum length (LE), scutum width (AE), basis capituli length (LBC), basis capituli width (ABC), gnathosoma length (LG), palp length (LP), length of right tarsus 1 (LT1D), width of right tarsus 1 (AT1D), maximum width of right spiracular plate (AMPED), width of the tail of the right spiracular plate (ACPED) and only in males the length of the right adanal plate (LPAD) and width of the right adanal plate (APAD) were measured.

Statistical Methods . Data obtained from Image - Pro ®Plus were exported for statistical analysis and calculation.

Principal Component Analysis (PCA). Principal component analysis (PCA) allows exploring the main axes of morphological variation in tangent space, observing individual variation trends and eventually recognizing particular groupings on a "factorial map", which is generally a "plot". of the first two principal components (Dujardin et al., 2014). This analysis does not take into account the belonging of each individual to different groups. It is a blind analysis and can often detect these groups from the individual values (Jombart et al., 2010) . The analysis was carried out using the statistical program R Core Team version 3.3.4 using the " prcomp" package (RStudio Team, 2017).

Canonical Variable Analysis (CVA). It is a multivariate analysis method that requires information about belonging to a group (population, sex, etc.) for each individual. The (CVA) is usually computed from the PCA values, its objective is to determine if there is a relationship between two groups of variables and the magnitude of that relationship. The analysis was carried out using the statistical program R Core Team version 3.3.4 using the "MASS" package (RStudio Team, 2017).

RESULTS

Taxonomic Identification. Morphologically, 28 ticks were identified (12 from human patients and 16 from canines). Ticks from human patients corresponded to the genera *Rhipicephalus* spp. (Table 1) and those of canines belong to the genus *Rhipicephalus* spp. (Table 2).

Phenotypic characterization of ticks from humans. All morphological characteristics (Figure 2) and measurements (in mm) of adult *Rhipicephalus* spp. are expressed in Table 3, including the mean, standard deviation and minimum and maximum values.

Phenotypic characterization of ticks from canines. All morphological characteristics (Figure 3) and measurements (in mm) of adult *Rhipicephalus* spp. are indicated in Table 4, including the mean, standard deviation and minimum and maximum values, and Figure 1.

Phenotypic comparison of ticks of the genus *Rhipicephalus* spp. All the morphological characteristics of the ticks obtained from both humans and canines are shown in Tables 3 and 4. The PCA results are shown in Figures 4 and 5, for the female and male specimens, respectively.

In the PCA of females (Fig. 4) the first main component (PC1) explains 83.2% of the total variance obtained, it is mainly due to the morphological traits (from highest to lowest): "idiosoma length" (LI), "width of the scutum" (AE), "width of the idiosoma" (LA) and "length of the scutum" (LE). The second principal component (PC2) explains 10.6% of the total variance obtained, it is mainly due to the morphological characteristics: "idiosoma width" (AI) and "scutum length" (LE). In general, 93.7% of the total variance observed is explained by the two main components. Thus, PC1 indicates that the specimens with the largest size of the idiosoma; while CP2 places the individuals with the longest scutum in the upper part and the individuals with the largest width of the idiosoma in the lower part.

In males (Fig. 5), the PC1 obtained from the PCA explains 81.1% of the total variance observed, it is mainly due to the morphological characteristics: “idiosoma length” (LI), “scutum length” (LE), “idiosoma width” (AI) and “scutum width” (AE). On the other hand, the PC2 explains 12.8% of the total variance obtained, it is mainly due to the phenotypic characteristics: “scutum width” (AE), “idiosoma width”, “idiosoma length” (LI) and “width of the right adanal plate” (APAD). In this way, 93.9% of the total variance observed is explained by the two main components. In summary, PC1 indicates that the specimens with the largest size of the idiosoma and scutum are located on the right and the opposite case on the left; while PC2 places the individuals with the longest scutum and idiosoma in the lower part and the individuals with the longest idiosoma in the upper part.

In the analysis of canonical variables, the canonical discriminant functions have significant values of Wilks ' λ ($P<0.001$). As there are two groups, there is only one function for both females (Y_H) and males (Y_M), these include 100% of the accumulated variation and are as follows:

Y_H = – 5.273IL + 4.272IA + 3.077EL + 2.191EA + 31.198BCL + 11.921BCA – 7.902GL + 9.290PL – 4.505TDL – 16.936TDA + 4.123PEAM RIGHT – 9.956PEAC RIGHT

Y_M = – 4.122IL – 0.420IA + 0.669EL + 11.173EA + 55.343BCL + 0.496BCA – 44.859GL – 13.619PL – 1.695TDL – 22.762TDA + 34.313PEAM RIGHT – 47.670PEAC RIGHT – 4.639PADAL6 + ADA

The application of discriminant analysis increased the ability of morphological characteristics to discriminate or clearly differentiate human ticks (La Rioja) from canine ticks (Valencia). The results of the classification according to the prediction of groups obtained with these discriminant functions are included in Tables 5 and 6.

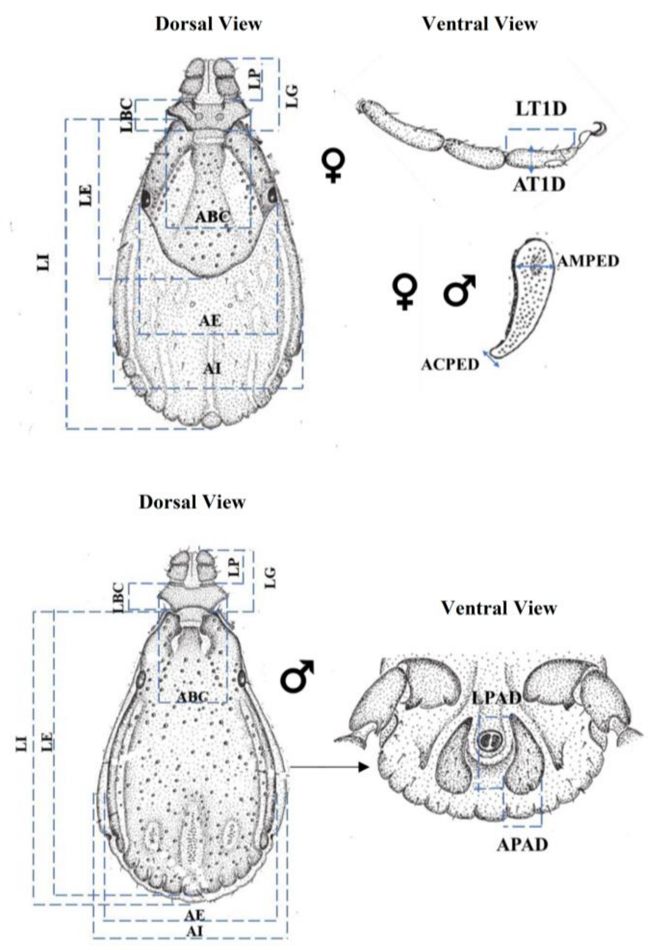

Figure 1. Measurement scheme of *Rhipicephalus* spp. ticks performed for morphometric analysis. Walker et al. (2003)

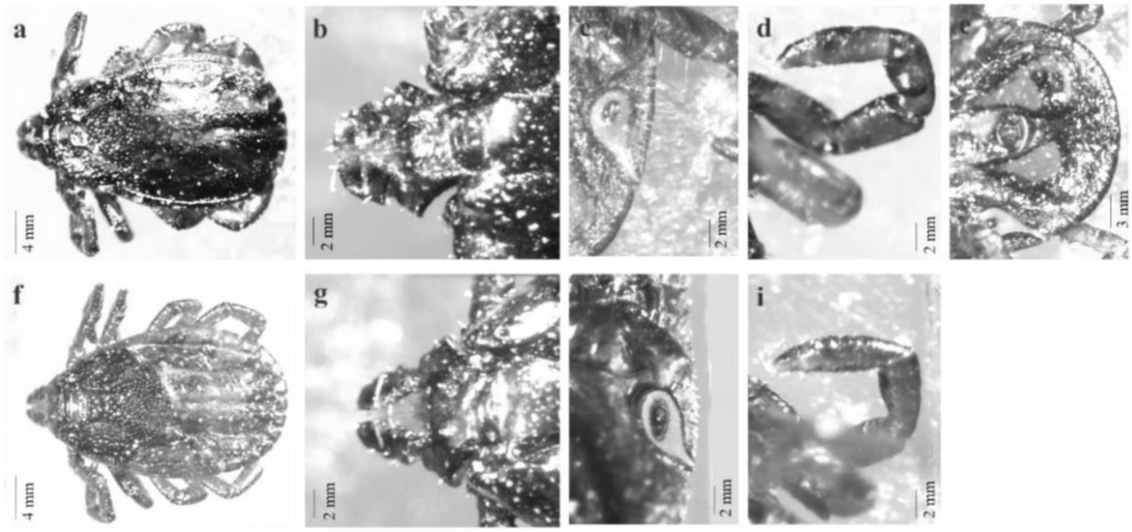

Figure 2. Morphological characteristics of ticks of the genus *Rhipicephalus* spp. of humans. **a, f** (scutum, idiosoma), **b, g (**gnathosoma, basis capituli, palps), **c, h** (spiracular plate), **d, i** (tarsus 1), **e** (adanal plates). References **a, b, c, d, e –** male and **f, g, h, i –** female.

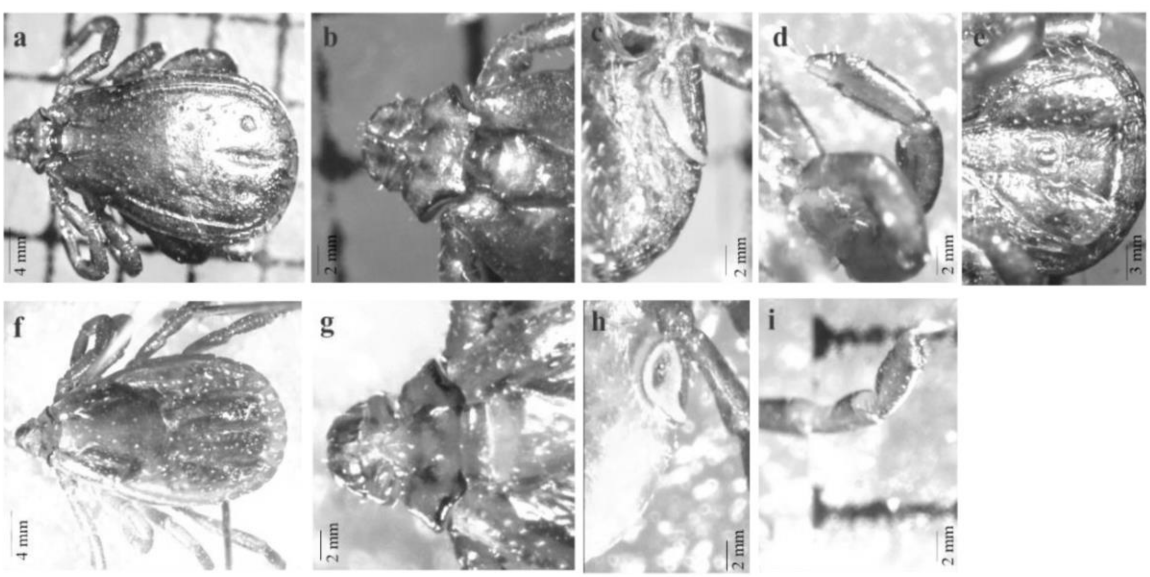

Figure 3. Morphological characteristics of ticks of the genus *Rhipicephalus* spp. of canines. **a, f** (scutum, idiosoma), **b, g (**gnathosoma, basis capituli, palps), **c, h** (spiracular plate), **d, i** (tarsus 1), **e** (adanal plates). References **a, b, c, d, e –** male y **f, g, h, i –** female

Table 1. List of specimens of the family Ixodidae (hard ticks) collected from human patients of the Riojano Health Service in La Rioja - Spain.

Genus/Species	Origin	Collection Date	Stage of life	Sex
***Rhipicephalus* sp.**	Logroño/La Rioja	2013	Adult	Male
***Rhipicephalus* sp.**	Logroño/La Rioja	2010	Adult	Male
***Rhipicephalus* sp.**	Logroño/La Rioja	2016	Adult	Male
***Rhipicephalus* sp.**	San Román de Cameros/La Rioja	2013	Adult	Female
***Rhipicephalus* sp.**	Logroño/La Rioja	2013	Adult	Male
***Rhipicephalus* sp.**	Logroño/La Rioja	2016	Adult	Male
***Rhipicephalus* sp.**	Logroño/La Rioja	2015	Adult	Male
***Rhipicephalus* sp.**	San Román de Cameros/La Rioja	2014	Adult	Female
***Rhipicephalus* sp.**	Logroño/La Rioja	2016	Adult	Female
***Rhipicephalus* sp.**	Logroño/La Rioja	2015	Adult	Male
***Rhipicephalus* sp.**	San Román de Cameros/La Rioja	2012	Adult	Male
***Rhipicephalus* sp.**	San Román de Cameros/La Rioja	2012	Adult	Female

Table 2. List of specimens of the family Ixodidae (hard ticks) collected from canines in La Cañada -Spain

Genus/Species	Origin	Capture Data	Collection Date	Stage of life	Sex
***Rhipicephalus* sp.**	Paterna/Valencia	canine	2012	Adult	Male
***Rhipicephalus* sp.**	Paterna/Valencia	canine	2012	Adult	Male
***Rhipicephalus* sp.**	Paterna/Valencia	canine	2012	Adult	Male
***Rhipicephalus* sp.**	Paterna/Valencia	canine	2012	Adult	Male
***Rhipicephalus* sp.**	Paterna/Valencia	canine	2012	Adult	Male
***Rhipicephalus* sp.**	Paterna/Valencia	canine	2012	Adult	Male
***Rhipicephalus* sp.**	Paterna/Valencia	canine	2012	Adult	Male
***Rhipicephalus* sp.**	Paterna/Valencia	canine	2012	Adult	Male
***Rhipicephalus* sp.**	Paterna/Valencia	canine	2012	Adult	Male
***Rhipicephalus* sp.**	Paterna/Valencia	canine	2012	Adult	Male
***Rhipicephalus* sp.**	Paterna/Valencia	canine	2012	Adult	Male
***Rhipicephalus* sp.**	Paterna/Valencia	canine	2012	Adult	Female
***Rhipicephalus* sp.**	Paterna/Valencia	canine	2012	Adult	Female
***Rhipicephalus* sp.**	Paterna/Valencia	canine	2012	Adult	Female
***Rhipicephalus* sp.**	Paterna/Valencia	canine	2012	Adult	Female
***Rhipicephalus* sp.**	Paterna/Valencia	canine	2012	Adult	Female

Table 3. Morphometric data of ticks *Rhipicephalus* spp. from humans (in mm).

Measures	Code	Males *Rhipicephalus* spp. *n=8	Females *Rhipicephalus* spp. n=4
IDIOSOMA LENGTH	LI	2.88 ± 0.33 2.25-3.34	3.41 ± 0.09 3.31-3.50
IDIOSOMA WIDTH	AI	2.00 ± 0.31 1.54-2.37	2.27 ± 0.20 2.08-2.54
SCUTUM LENGTH	LE	2.72 ± 0.29 2.20-3.13	1.64 ± 0.09 1.53-1.74
SCUTUM WIDTH	AE	1.74 ± 0.26 1.28-2.07	1.60 ± 0.07 1.54-1.70
BASIS CAPITULI LENGTH	LBC	0.28 ± 0.07 0.19-0.37	0.32 ± 0.02 0.28-0.34
BASIS CAPITULI WIDTH	ABC	0.67 ± 0.07 0.55-0.76	0.85 ± 0.02 0.82-0.88
GNATHOSOMA LENGTH	LG	0.56 ± 0.10 0.40-0.67	0.69 ±0.05 0.64-0.76
PALP LENGTH	LP	0.38 ± 0.07 0.27-0.50	0.50 ± 0.01 0.48-0.51
LENGTH OF RIGHT TARSUS 1	LT1D	0.56 ± 0.09 0.39-0.67	0.66 ± 0.06 0.60-0.72
WIDTH OF RIGHT TARSUS 1	AT1D	0.18 ± 0.03 0.11-0.21	0,19 ± 0,01 0.17-0.20
MAXIMUM WIDTH OF RIGHT SPIRACULAR PLATE	AMPED	0.26 ± 0.03 0.23-0.32	0.29 ± 0.06 0.22-0.36
WIDTH OF THE TAIL OF THE RIGHT SPIRACULAR PLATE	ACPED	0.04 ± 0.02 0.03-0.08	0.06 ±0.02 0.04-0.08
LENGTH OF THE RIGHT ADANAL PLATE	LPAD	0.82 ± 0.16 0.54-1.11	--
WIDTH OF THE RIGHT ADANAL PLATE	APAD	0.50 ± 0.14 0.26-0.71	--

Table 5. Results of the prediction classification of groups of female ticks.

Current group	N°	Prediction	
		Human ticks La Rioja (GR)	Canine ticks Valencia (GV)
Human ticks La Rioja (GR)	**4**	**4 (100%)**	**0**
Canine ticks Valencia (GV)	**5**	**0**	**5 (100%)**

Table 6. Results of the prediction classification of groups of male ticks.

Current group	N°	Prediction	
		Human ticks La Rioja (GR)	Canine ticks Valencia (GV)
Human ticks La Rioja (GR)	**8**	**8 (100%)**	**0**
Canine ticks Valencia (GV)	**11**	**0**	**11 (100%)**

DISCUSSION

Morphometry applied to medical entomology aims to help systematic research and quantify the phenotypic variation of an organism (Dujardin and Slice, 2007) . Morphometry has been frequently used in acarological studies in systematics, for the validation of intraspecific variability, establishment of synonyms in the definition of useful traits in the systematics of genera and families, for which several studies have been developed in vectors such as ticks focused in traditional morphometric analyses (Dantas-Torres et al., 2013; Coimbra-Dores et al., 2016; Nava et al., 2016; Sanches et al., 2016) ; as well as molecular studies based on mitochondrial genes (Mangold et al., 1998b; Latrofa et al., 2013; Hekimoğlu et al., 2016), which have made it possible to clarify their taxonomy.

Analysis of the morphometric results. The PCA, carried out with morphometric traits, showed a clear separation between female ticks of Rhipicephalus spp. of humans with Rhipicephalus spp. of dogs, mainly explained by the length of the idiosoma and width of the scutum, in the same way a separation between male ticks of Rhipicephalus spp. of humans with Rhipicephalus spp. of canines, explained by the length of the idiosoma and length of the scutum. Several studies indicate that the tick which mainly affects dogs is Rhipicephalus sanguineus, commonly known as the "brown dog tick", which parasitizes many vertebrates, including occasionally humans, has a worldwide distribution and is a vector of recognized importance for public health. (Dantas-Torres et al ., 2013; Abdullah et al., 2016) .Sanches et al. (2016) compared different populations of *Rhipicephalus sanguineus sensu lato* (*sl*) by morphometric and molecular methods. Their morphometric analysis revealed a clear separation between females from Brazil (Jaboticabal, SP), Cuba (Havana) Thailand (Bangkok), called "tropical strain" ticks, and female ticks from Spain (Zaragoza), Argentina (Rafaela, Santa Fe), called "temperate strain" ticks. Likewise, males exhibited the same separation, where PCA, between tropical strains and temperate strains of both male and female ticks was explained by idiosoma length and width. Their molecular analysis corroborated the existence of two different clades belonging to tropical strains and temperate strains. Some morphological variations confirmed the existence of at least two species under the name of *R. sanguineus* (Sanches et al., 2016).

Coimbra-Dores et al. (2016), morphometrically compared specimens collected in dogs where the highest percentage were ticks of the species *R. sanguineus*, followed by *Rhipicephalus turanicus* and to a lesser extent by *Rhipicephalus pusillus*. Its PCA, obtained for both genera showed an overlap between the specimens that were identified as *R. sanguineus* and *R. turanicus*, while *R. pusillus* formed an isolated group. As for females, their CP1 was explained by the width of the chapter base and the length of the palps, while in males their CP1 was explained by the length and width of the idiosoma. Curiously, some specimens were classified as intermediate due to their morphological variations and were found in the PCA in the intermediate zone between *R. sanguineus* and *R. turanicus*.

R. sanguineus sensu stricto (*ss*) ticks, are a species with considerable public health importance. However, the taxonomic status of this species is far from resolved. After several years of scientific work on *R. sanguineus s.s.*, the situation is that there is no solid description, nor is there consensus on the range of morphological variability within the species. It lacks an original informative description and is based on the existence of several morphological descriptions based on ticks originating from different populations, which present significant genetic divergence (Nava et al ., 2015)

The *R. sanguineus* complex includes 17 species that are morphologically similar and consequently have been misidentified (Dantas-Torres & Otranto, 2015; Sanches et al ., 2016).

Interestingly, a study carried out by Fernández (2003), in Castilla y León from 1997 to 2002, collected and identified a total of 3059 ticks from 2717 humans, all the ticks belonged to 15 species (14 ixodid and 1 argasid). The most abundant species was *I. ricinus* with 1320 specimens, followed in abundance by *R. bursa*, *Dermacentor marginatus*, *R. turanicus* and *H. marginatum.* The species with a lower frequency was *R. sanguineus*, *H. lusitanicum* and *R. pusillus*. It should be noted that 10.68% of these ticks were carriers of pathogenic bacteria. The distribution of the species was associated to zones such as: *I. ricinus* in mountainous areas of the northern and southern provinces; *R. bursa* and *H. marginatum* in dehesa areas of Ávila, Salamanca and Zamora; *R turanicus* and *R. sanguineus* anywhere where dogs, cattle or sheep are present; *H. lusinaticum* on southern slopes. In addition, it should be said that *I. ricinus* has a great tendency to bite humans, unlike *R. sanguineus*, which shows a low tendency, while that of the rest of the species remains intermediate between the two. Depending on their evolutionary phase, adult *H. marginatum* males have a propensity to parasitize humans.

The diversity of tick species in Spain is of interest at the level of human and animal health and its distribution is perfectly marked due to its climate preferences (Gray et al ., 2009). *Ixodes ricinus,* in Spain, is distributed throughout the country, preferably in cool and humid regions of the northern peninsula, *R. sanguineus*, *R. bursa* and *R. turanicus* are also distributed throughout the country, while *R. pusillus* and *R. (Boophilus) annulatus* are encountered in regions with a Mediterranean climate. Similarly, the species of *Hyalomma* present in Spain are distributed in regions with a Mediterranean climate, where the species *H. marginatum* prefers areas with relatively high temperatures in summer and can tolerate low values of environmental humidity (Estrada-Peña, 2015). For this reason, the number of diseases transmitted by ticks varies depending on the geographical area and depending on the ticks that circulate in that area (Barandika et al ., 2011). Pathogens transmitted by ticks represent a public health problem. This fact highlights the importance of characterizing ticks that parasitize humans and animals.

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REFERENCES CITED

Abdullah H., El-Molla A., Salib F., Allam N., Ghazy A. and Abdel-Shafy S.. 2016.- Morphological and molecular identification of the brown dog tick *Rhipicephalus sanguineus* and the camel tick *Hyalomma dromedarii* (Acari: Ixodidae) vectors of Rickettsioses in Egypt. Veterinary World, 9 (10): 1087–1101.

Barandika J., Hurtado A., Juste R. and García-Pérez A. 2010.- Seasonal dynamics of *Ixodes ricinus* in a 3-year period in northern Spain: First survey on the presence of tick-borne Encephalitis Virus. Vector-Borne and Zoonotic Diseases, 10 (10): 1027-1035.

Barandika J., Olmeda S., Casado-Nistal M., Hurtado A., Juste R., Valcárcel F. and García-Pérez A.. 2011. -Differences in questing tick species distribution between Atlantic and continental climate regions in Spain. Journal of Medical Entomology, 48 (1): 13–19.

Coimbra-Dores M., Nunes T., Dias D. and Rosa F.. 2016.- *Rhipicephalus sanguineus* (Acari: Ixodidae) species complex: morphometric and ultrastructural analyses. Experimental and Applied Acarology, 70 (4): 455–468.

Dantas-Torres (F.), Latrofa S., Annoscia G., Giannelli A., Parisi A. and Otranto D. 2013.- Morphological and genetic diversity of *Rhipicephalus sanguineus sensu lato* from the New and Old Worlds. Parasites & Vectors, 6 (1): 213.

Dantas-Torres F. and Otranto D. 2015.- Further thoughts on the taxonomy and vector role of *Rhipicephalu s sanguineus* group ticks. Veterinary Parasitology, 208 (1): 9–13.

Dujardin J. P. and Slice D. E. 2007.- Contributions of morphometrics to medical entomology. En: *Encyclopedia of Infectious Diseases*: *Modern Methodologies.* (M. Tibayrenc, ed.). Hoboken: John Wiley & Sons. pp. 433–445.

Dujardin J. P., Kaba D., Solano P., Dupraz M., McCoy K. and Jaramillo O. 2014.- Outline-based morphometrics, an overlooked method in arthropod studies. Infection, Genetics and Evolution, 28: 704–714.

Estrada-Peña A. and Jongejan F. 1999.- Ticks feeding on humans: A review of records on human-biting Ixodidae with special reference to pathogen transmission. Experimental and Applied Acarology, 23 (9): 685–715.

Estrada-Peña A. 2015.- Orden Ixodida: Las garrapatas. Revista IDEA-SEA, 13: 30–6.

De La Fuente J., Naranjo V., Ruiz-Fons F., Vicente J., Estrada-Peña A., Almazán, C. and Gortázar, C. 2004 Prevalence of tick-borne pathogens in ixodid ticks (Acari: Ixodidae) collected from European wild boar (*Sus scrofa*) and Iberian red deer (*Cervus elaphus hispanicus*) in central Spain. European Journal of Wildlife Research, 50 (4): 187–196.

Fernández P. 2003.- Garrapatas que parasitan a las personas en Castilla y León , determinación por serología de su parasitismo y detección moleculaza de los patógenos que albergan. (Tesis doctoral). Universidad de Salamanca, España.

Gray J., Dautel H., Estrada-Pena A., Kahl, O. and Lindgren E. 2009.- Effects of climate change on ticks and tick-borne diseases in Europe. Interdisciplinary Perspectives on Infectious Diseases,2009.

Hekimoğlu O., Sağlam I., Özer N. and Estrada-Peña A. 2016.- New molecular data shed light on the global phylogeny and species limits of the *Rhipicephalus sanguineus* complex. Ticks and Tick-Borne Diseases, 7 (5): 798–807.

Jombart T. Devillard S. & Balloux F. 2010.- Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. BMC Genetics, 11 (1): 94.

Latrofa, M. S., Dantas-Torres, F., Annoscia, G., Cantacessi, C., & Otranto, D. 2013. Comparative analyses of mitochondrial and nuclear genetic markers for the molecular identification of *Rhipicephalus* spp. Infection, Genetics and Evolution, 20: 422–427.

Mangeaud A. 2004.- La aplicación de técnicas de ordenación multivariadas en la Entomología. Revista de la Sociedad Entomológica de Argentina, 63 (3–4): 1–10.

Mangold A., Bargues M. D. and Mas-Coma S. 1998a.- 18S rRNA gene sequences and phylogenetic relationships of European hard- tick species (Acari: Ixodidae). Parasitology Research, 84 (1): 1–37.

Mangold A., Bargues M. D. and Mas-Coma S. 1998b.- Mitochondrial 16S rDNA sequences and phylogenetic relationships of species of *Rhipicephalus* and other tick genera among Metastriata (Acari: Ixodidae). Parasitology Research, 84 (6): 478–484.

Márquez-Jiménez F., Hidalgo-Pontiveros A., Contreras-Chova F., Rodríguez-Liébana J., and Muniain-Ezcurra A. 2005.- Las garrapatas (Acarina: Ixodida) como transmisores y reservorios de microorganismos patógenos en España. Enfermedades Infecciosas y Microbiología Clínica, 23 (2): 94–102.

Nava S., Estrada-Peña A., Petney T., Beati L., Labruna M., Szabó, M. and Guglielmone A.. 2015.- The taxonomic status of *Rhipicephalus sanguineus* (Latreille, 1806). Veterinary Parasitology, 208 2–8.

Nava S., Gerardi M., Szabó M., Mastropaolo M., Martins T., Labruna M., Beati L., Estrada-Peña A. and Guglielmone A. 2016.- Different lines of evidence used to delimit species in ticks: A study of the South American populations of *Amblyomma parvum* (Acari: Ixodidae). Ticks and Tick-Borne Diseases, 7 (6): 1168–1179.

Norris D., Klompen J. and Black W..1998.- Comparison of the mitochondrial *12S* and *16S* ribosomal DNA genes in resolving phylogenetic relationships among hard ticks (Acari: Ixodidae). Annals of the Entomological Society of America, 92 (1): 117–129.

Organización Mundial de la Salud (OMS). 2016.- Enfermedades transmitidas por vectores. [actualizado 5 de febrero de 2016; acceso 3 de marzo de 2017]. Disponible en: http://www.who.int.

Palomar A., Portillo A., Santibá S., Márquez F., Romero L., Eiros J. and Oteo J. 2017.- Molecular (ticks) and serological (humans) study of Crimean-Congo hemorrhagic fever virus in the Iberian Peninsula. Enfermedades Infecciosas y Microbiologia Clinica, 3 (6): 344–347.

RStudio Team (RStudio). 2017. – RStudio: Integrated Development for R. RStudio. [actualizado 25 de marzo de 2017; acceso 10 de junio de 2017]. Disponible en: https://www.rstudio.com.

Sanches G., Évora P., Mangold A., Jittapalapong S., Rodriguez-Mallon A., Guzmán P. and Camargo-Mathias M. . 2016.- Molecular, biological, and morphometric comparisons between different geographical populations of *Rhipicephalus sanguineus* sensu lato (Acari: Ixodidae). Veterinary Parasitology, 215: 78–87.

Walker A., Bouattour A., Camicas J., Estrada-Peña A., Horak I., Latif A., Pegram R., Preston P. (eds.). 2003.- Ticks of domestic animals in Africa : a guide to identification of species. Edinburgh: Bioscience reports. 221 pp.