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Molecular and serological prevalence of *Anaplasma marginale* in cattle of North Central Morocco

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ABSTRACT

A cross sectional study was conducted to investigate the epidemiological distribution of *Anaplasma marginale* in North Central Morocco. Blood samples from five provinces of Morocco were collected from apparently healthy cattle ($n = 668$) and simultaneously analyzed by a nested polymerase chain reaction (nPCR) assay and competitive enzyme-linked immunosorbent assay (cELISA). The overall prevalence of *A. marginale* was 21.9% by nPCR and 16.5% by cELISA. The Kappa coefficient between nPCR and cELISA indicated a modest level of agreement (0.54). The prevalence of *A. marginale* varied significantly according to the province and the month of sampling. However age, gender and breed did not have a significant effect on the prevalence of this pathogen. The highest prevalence of *A. marginale* was found in the Gharb, a sub-humid area while the lowest was reported in the Saiss, a semi-arid area. These results indicate that an *A. marginale* infection are widespread in the country and suggests that either or both techniques are excellent tools for epidemiological studies and control programs.

1. Introduction

Bovine anaplasmosis is a hemoparasitic disease caused by the tick-borne pathogen *Anaplasma marginale* (Dumler et al., 2001) and *Anaplasma centrale* (Inokuma et al., 2001). Anaplasma is globally the most prevalent tick-borne pathogen of cattle and has a worldwide distribution with regions of endemicity on six continents (Futse et al., 2003) and high incidence in tropical and subtropical regions of the world (De La Fuente et al., 2005; Torina et al., 2008; Gokce et al., 2008; Ooshiro et al., 2009; Ruybal et al., 2009). *A. centrale* is widely distributed but has never been reported in North Africa, it has been reported only in South Africa (OIE, 2008). However, the geographic distribution depends largely on the distribution and density of the reservoir hosts and tick vectors (Ogden et al., 2002).

Biological transmission of *A. marginale* is effected by ticks and approximately 20 species of ticks have been incriminated as vectors worldwide, including *Boophilus* spp., *Rhipicephalus* spp., *Hyalomma* spp., *Demacentor* spp. and *Ixodes* spp. (Uilenberg, 1995; De Waal et al., 2000; Jongejan and Uilenberg, 2004; Kocan et al., 2004). Tick transmissibility of *Anaplasma* is complex and ranges from very efficient transmission to non-transmission related to both *Anaplasma* strains as well as species of tick (Ueti et al., 2007). In Morocco, the vector competency of local tick species for transmitting *A. marginale* has yet to be demonstrated.

Following transmission, cattle develop rickettsiaemia, accompanied by fever, severe anemia, weight loss, decreased milk production, abortion and sometimes death during acute infections (Kocan et al., 2003; Urdaz-Rodríguez et al., 2009). After the first infection with *A. marginale*, cattle remain persistently infected carriers and serve as long-term reservoirs for the maintenance of the infection in ticks (Goff et al., 1988; Kocan et al., 1992a,b; Eriks et al., 1993). Anaplasmosis has been reported in different regions of Morocco (Verhulst et al., 1983; Sahibi et al., 1998b), but it has a perceived lower incidence compared with theileriosis and babesiosis. Although, severe economic losses have been reported due to anaplasmosis outbreaks in several parts of the world (Herrero et al., 1998; Kocan et al., 2000, 2004; Grisi et al., 2002; Jongejan and Uilenberg, 2004), no specific disease outbreak of *A. marginale* in cattle has been reported in Morocco and the only epidemiological studies of anaplasmosis reported previously were conducted more than a decade ago (Sahibi et al., 1998b).

Diagnosis of the disease is usually based on microscopic examination of stained blood films; however this method can only detect levels of 10^6 infected erythrocytes per ml (Gale et al., 1996) in acute infection but it is not sufficiently sensitive or specific to detect chronic carriers. A cELISA was developed based on antibody

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binding to recombinant *A. marginale* major surface protein 5 (msp5) (Knowles et al., 1996). Msp5 is a protein conserved among *Anaplasma* spp. This assay was used to detect both acute and chronic *A. marginale* and *A. centrale* infections in cattle (Knowles et al., 1996), *A. ovis* infections in goats (Ndung'u et al., 1995) and sheep and wildlife (Scoles et al., 2008). Recently, molecular methods based on nucleic acids such as nPCR offer greater sensitivity and specificity over the existing diagnostic tests (Torioni de Echaide et al., 1998). Consequently a combined nPCR/cELISA approach could provide powerful tools for epidemiological investigations with high accuracy in the diagnosis of Anaplasma infections. Successful management of anaplasmosis in cattle depends on adequate knowledge of prevalence for *A. marginale* and the risk factors associated with transmission (Alonso et al., 1992; Swai et al., 2005). The aim of this study was to determine the prevalence and risk factors of anaplasmosis by cELISA and nPCR. This study formed a part of an epidemiological survey of selected tick-borne diseases in five provinces of Morocco.

2. Materials and methods

2.1. Area of study

A cross sectional study was carried out between March and August 2005 in five provinces of North Central Morocco: Kénitra (34°15′N 6°35′W), Sidi Slimane (34°13′N 5°42′W), Sidi Kacem (34°13′N 5°42′W), Meknes (33°53′N 5°33′W) and El Hajeb (33 51′N 7 02′W). These study areas belong to three ecological zones: Gharb, Saiss and Middle Atlas (Fig. 1).

2.1.1. Region of Gharb

This region includes Kénitra, Sidi Slimane and Sidi Kacem provinces.

Kénitra province is located at an altitude of 25 m and has a subhumid climate with a mean annual rainfall of 600 mm. The minimum average temperature of the coldest month is about 5 °C and the maximum average temperature of the hottest month is about 30 °C.

Sidi Slimane is located at an altitude of 41 m, and has a semi-arid climate with a mean annual rainfall of 500 mm. The minimum average temperature of the coldest month is about 4 °C and the maximum average temperature of the hottest month is about 35 °C.

Sidi Kacem is located at an altitude of 74 m, and has a semi-arid climate with a mean annual rainfall of 500 mm. The minimum average temperature of the coldest month is about 5 °C and the maximum average temperature of the hottest month is about 35 °C.

2.1.2. Region of Saiss

This region includes Meknes province which ranges from 145 to 549 m in altitude and has a semi-arid climate with a mean annual rainfall of 545 mm. The minimum average temperature of the coldest month is 4 °C and the maximum average temperature of the hottest month is about 35 °C.

2.1.3. Region of the Middle Atlas

This region includes El Hajeb province where the climate is humid with cold winters in the higher elevations. The effect of altitude (about 1200 m) decreases the temperatures: winter is very cold, summer is moderate. Rainfall is about 1200 mm, and occurs between October and April. From May to September the weather is hot and dry with a minimum average temperature of the coldest month of about −4 °C and the maximum average temperature of the hottest month of about 30 °C.

The farmers in these regions practice an agro-pastoral farming system which is the main source of milk and meat consumed in Morocco (Guessous, 1991). *Hyalomma detritum, Hyalomma marginatum*, and *Rhipicephalus* (*Boophilus*) *annulatus* are the major tick species of economic importance in the study area (El Kamch, 2005).

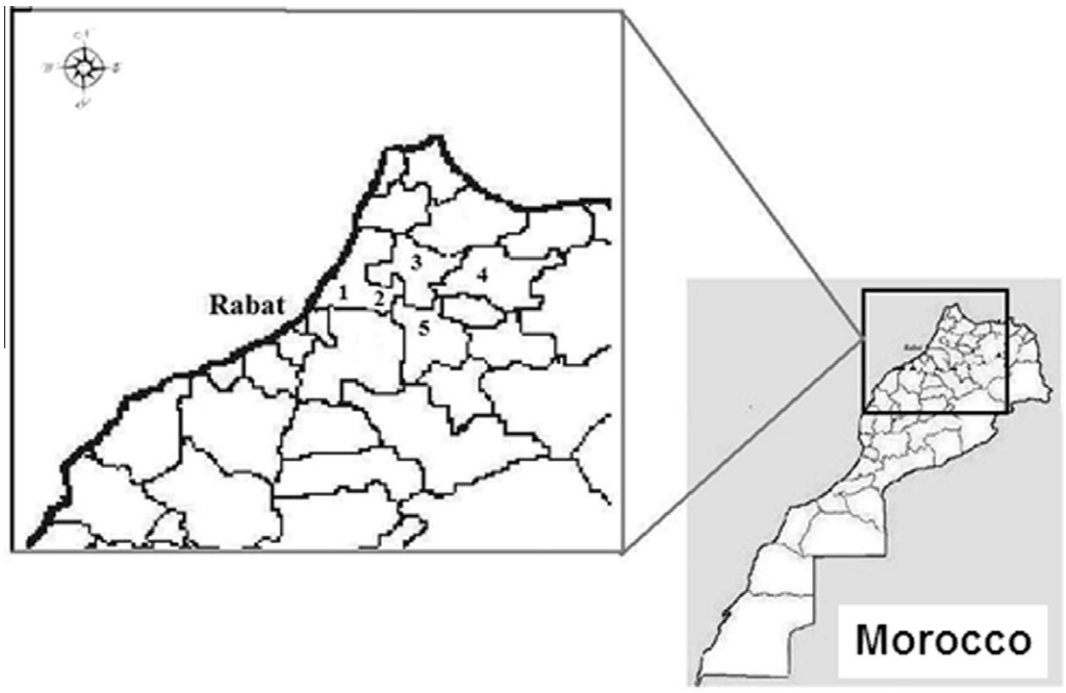

Fig. 1. Study area: Region of Gharb: (1) Kénitra province; (2) SidiSlimane province; (3) SidiKacem province; Region of Saiss: (4) Meknes province; and Region of Middle Atlas: (5) El Hajeb province.

2.2. Collection of blood samples

Farms were visited twice a month in each site and a total of 668 cattle were randomly sampled (252 males and 416 females). Cattle sera were collected from various breeds, which included local (39.7%), imported (24.1%) and cross-breed (36.2%).

Blood was collected into sterile tubes with and without anticoagulant (EDTA) and maintained at 4 °C until arrival at the laboratory. Serum was separated, and serum and whole blood were stored at −20 °C.

2.3. Anaplasmosis serological test

The anaplasmosis cELISA was performed using the *Anaplasma* Antibody Test Kit, cELISA from VMRD Inc. (Pullman, WA, USA) following the manufacturer's instructions. This assay detects serum antibodies against the Msp5 protein of *Anaplasma* spp. (Knowles et al., 1996).

2.4. DNA extraction

Blood collected on EDTA was washed three times with PBS buffer to remove the leukocyte layer. The resulting red blood cell mass was processed to extract the DNA according to the manufacturer's recommendations (Puregene kit Gentra Systems Inc.). The extracted DNA was kept refrigerated (4 °C) until the nPCR analysis was carried out.

2.5. Nested PCR

The nPCR was performed on the extracted DNA by using primers specific for the *msp5* gene as described by Torioni de Echaide et al. (1998). *Msp5* was amplified using the following primers: external forward 5′-GCATAGCCTCCGCGTCTTTC-3′ and external reverse 5′-TCCTCGCCTTGGCCCTCAGA-3′ and the internal forward primer 5′-TACACGTGCCCTACCGAGTTA-3′. Both reactions were performed under the same cycling conditions with a GeneAmp 2400 system (Applied Biosystems Inc.) beginning with a hot start for 3 min at 95 °C followed by 35 cycles with denaturation for 30 s at 95 °C, annealing for 58 s at 65 °C, and extension for 30 s at 72 °C and a final extension for 10 min at 72 °C. The product of the primary PCR was then run under the same conditions for an additional 35 cycles with the appropriate primers for the secondary PCR. Genomic DNA of *A. marginale* was isolated from infected blood showing high rickettsiaemia during blood smear examination and utilized as a positive control. Blood from Cattle shown to be free of *A. marginale* by subinoculation of a blood sample into a splenectomized calf was used as a negative control. Controls were also subjected to the same treatment.

Products were visualized in a 1.5% agarose gel containing ethidium bromide and visualized by UV transillumination. A 457-bp band is expected after the primary PCR and a 345-bp band is expected after nested PCR. The 345-bp expected DNA fragment was identified by comparison with 100 base pair incremental molecular markers (100 bp Ladder, Gibco BRL) (Fig. 2).

2.6. Statistical analysis

Data files were entered, edited and performed on Epi-Info 2007 (version 3.4). Bivariant analysis was used to assess the relationship between Anaplasma seropositive animals and the risk factors responsible for the infection. The chi-square test was used to evaluate significant differences of infection rate in animals of different gender, age, season and location. *P* values for significance were set at $P \leqslant 0.05$. The kappa coefficient was calculated to evaluate the degree of agreement between the nPCR assay and cELISA.

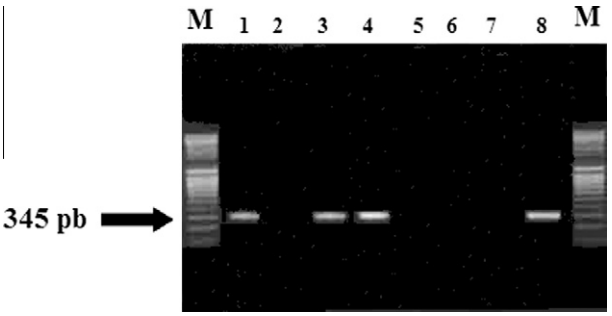

Fig. 2. Agarose-gel electrophoresis of amplification product obtained from *Anaplasma marginale* using *Anaplasma* specific primers. Lines M: molecular weight (100bps); line 1–3 and 4: blood from positive cattle; line 2–5 and 6: blood from negative cattle; line 7: negative control; line 8: positive control.

3. Results

During a period of five months, 668 cattle blood samples (252 male and 416 female) were randomly collected from the five different provinces identified above in North Central Morocco and simultaneously analyzed by nPCR and cELISA techniques. The mean age was 17.48 ± 11.03 months and the sex ratio (male/female) was 0.60.

When the two assays were compared, there was an 85.6% concordance with 81 samples positive and 491 samples were negative by both assays (Table 1). The cELISA identified 112 positive samples or a prevalence rate of 16.8% while the nPCR identified 146 positive samples or a prevalence of 21.9% (Table 1). Sixty-five samples were nPCR positive and serologically negative and 31 samples were serologically positive and nPCR negative.

Although there was an 85.6% concordance, nPCR detected higher positive rates of the parasite than the cELISA. The kappa value was fairly low (0.54) (Table 1).

The prevalence rate in North Central Morocco may be as high as 26.5% using the total number of samples that were positive for either test. The prevalence of bovine anaplasmosis in different cattle populations was compared on the basis of different age, gender, breed, month, climate and province again using the total number of samples positive for either test (Fig. 3). There was no statistically significant association for the prevalence of *A. marginale* among different age groups ($X^2 = 0.59$, $P > 0.05$), and no significant difference observed between gender ($X^2 = 0.34$, $P > 0.05$) nor breed ($X^2 = 0.82$, $P > 0.05$) of cattle. The highest prevalence of *A. marginale* was observed in Kenitra with 52%, which was significantly higher compared with the other sites ($X^2 = 78.22$, $P < 0.05$). Furthermore, there were statistically significant differences between the climates and prevalence of *A. marginale*. The infection rates of *A. marginale* was significantly higher in August and June compared to other months ($X^2 = 28.27$, $P > 0.05$) (Fig. 3).

4. Discussion

Despite the fact that several studies have reported on the presence of tick-borne diseases in Morocco (Ouhelli and Flach, 1990; Sahibi et al., 1998a, 1998b; El Haj et al., 2002; Sahibi and Rhalem, 2007), there is still little information about the prevalence of anaplasmosis. In this study we investigated the epidemiology of *A. marginale* infections in cattle from five provinces of North Central Morocco using the nPCR and cELISA. The results clearly indicated the presence of *A. marginale* infection in this region of the country with an overall prevalence of 26.5%. A few previous serological studies involving *A. marginale* reported that the seroprevalence ranged from 9% to 22.2% in different regions of Morocco

Table 1
Summary of the molecular and serological detection of *A marginale* using the nPCR assay and cELISA among 668 cattle sampled in North Central Morocco.

nPCR[a]	cELISA[b]				Total	PCR/cELISA[c]	% Agreement[d] (Kappa value)
	Positive (%)		Négative (%)				
Positive	81	(72,3)	65	(11,7)	146	177	85.6 (0,54)
Négative	31	(27,7)	491	(88,3)	522	491	
Total	112		556			668	

[a] The frequency of positive and negative samples as results of nPCR.
[b] The frequency of positive and negative samples as result of cELISA cross – tabulated with nPCR.
[c] The frequency of positive and negative samples of combined nPCR and ELISA.
[d] Concordance between nPCR and cELISA results.

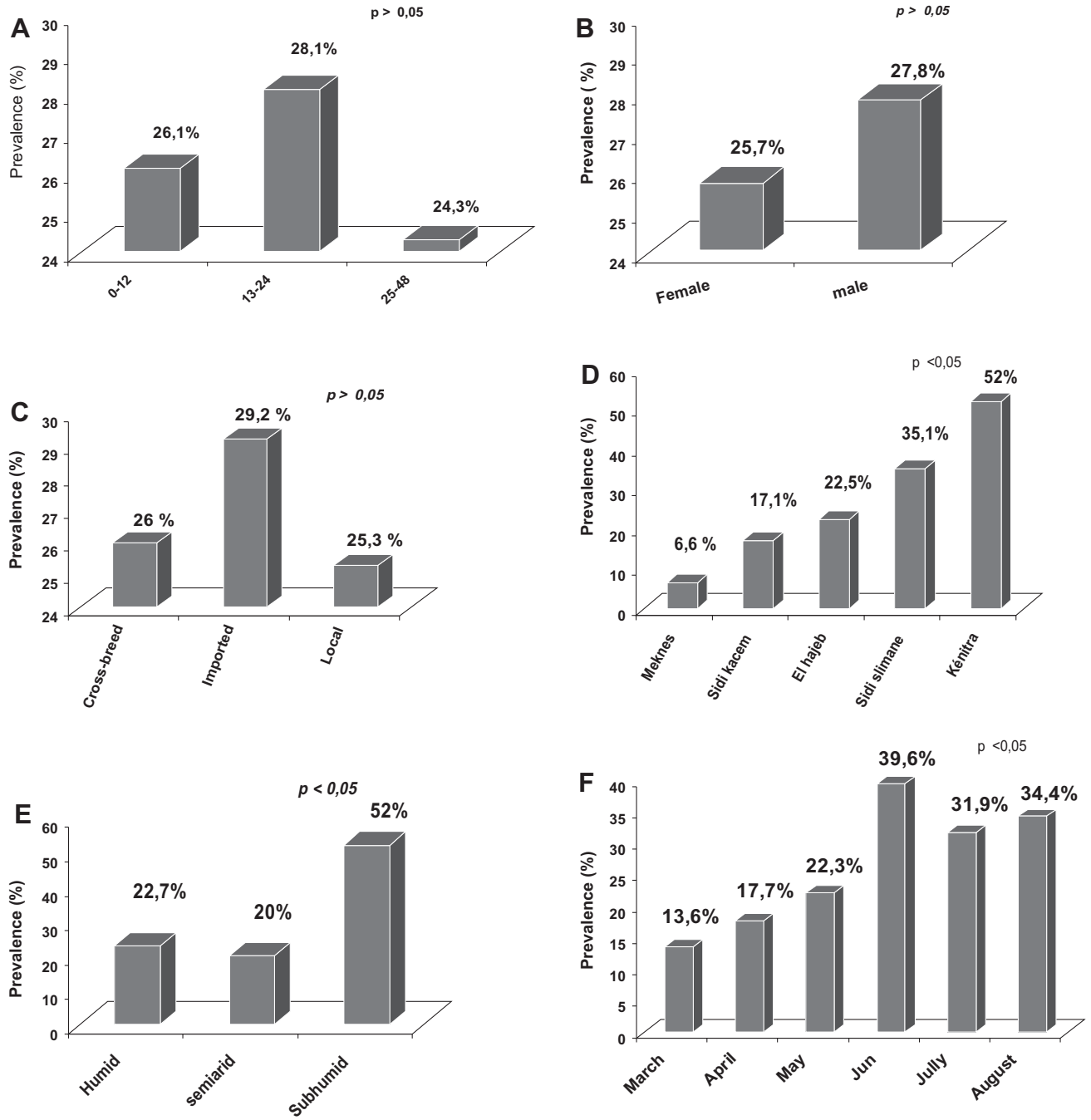

Fig. 3. Comparison of the overall prevalence of *A. marginale* on the basis of age (A), gender (B), breed (C), provinces (D), climate (E) and month of sampling (F).

(Verhulst et al., 1983; Sahibi et al., 1998b; Sahibi and Rhalem, 2007). Compared to other North African Mediterranean countries, the prevalence in Morocco remains higher than those observed in Algeria 7.4% (Ziam and Benaouf, 2004) and in Egypt 3.5% (Younis et al., 2009). Moreover, our finding was similar to that observed in Puerto Rico with 27.4% (Urdaz-Rodríguez et al., 2009) and Tanzania (37.2%) (Swai et al., 2005). In other regions anaplasmosis prevalence occurs at much higher levels i.e. in Kenya (89%) (Maloo et al., 2001), El Salvador (78.5%) (Payne and Scott, 1982) and St. Lucia (70%) (Hugh-Jones et al., 1988). However, prevalence rates reported for countries must be taken with caution since a standardized assay as sampling procedure was not used in each study and rates of infection may vary even among neighboring farms (Stuen et al., 2002).

The prevalence of *Anaplasma* infection is affected by the geographic situation and other risk factors. In our study, we investigated the relationship between risk factors such as age, gender and different breeds, but only the province, climate and month of sampling demonstrated significant effects. The positive animals were found in all provinces investigated during the period of survey. The highest prevalence of *A. marginale* was observed in Kenitra (52%) in contrast to previous reports of 10.6% (Sahibi et al., 1998b). In the current study a lower prevalence was reported in Meknes (Saiss) with 6.6% which is similar to the previous report of 9% in the same area (Sahibi et al., 1998b).

In general, seropositivity in cattle decreased while going from the Gharb toward the Middle Atlas regions suggesting that the change of climate and vegetation cover are important factors limiting the development of the tick vectors. This result was expected as it is in agreement with described geographic tick distribution in the region (Sahibi and Rhalem, 2007).This result is also supported by the fact that *Rhipicephalus* (*Boophilus*) *annulatus* is a known vector of *A. marginale* (Scoles et al., 2008) and its prevalence in the Gharb region is 34% (Sahibi and Rhalem, 2007). This tick species is far less abundant in Saiss and Middle Atlas (El Kamch, 2005). In the current study, the sub-humid climate zone showed the highest percentage of infected cattle (64/123, 52%). The semi-arid climate of Meknes showed the lowest prevalence (6%). However in the province of Sidi Slimane with a similar climate, *A. marginale* prevalence was 35.1%. Thus, the significant differences in prevalence cannot be explained strictly by ecology or the dynamics of the tick population in the different zones. Other components such as immunity level, tick control practices, tick species and grazing system are likely to play a role as well.

In all areas there was a clear pattern of increased numbers of positive samples during the summer season (June, July and August) which likely corresponds with increased tick activity in the preceding months. Vector populations are associated with many factors of which climate, season and host availability are probably the most important.

There is no certainty that there is an absence of clinical signs of anaplasmosis in this area since diagnosis based on nonspecific clinical signs (fever and anemia) could lead to misdiagnosis with other diseases, such as babesiosis and theileriosis. However, practitioners in the region have not reported what they think to be clinical anaplasmosis.

If this is the case, a long-term endemicity of infection may be present in the studied areas with a substantial frequency of asymptomatic chronic carriers.

The overall prevalence of *A. marginale* infections was compared among different cattle on the basis of age gender and breed. Somewhat surprising, there was no significant difference in age, in contrast to a previous study from Sudan (Awad et al., 2011). Cattle of all ages are susceptible to anaplasmosis but the severity of the infection is directly related to age, with older animals suffering more severe clinical disease. Indeed, in this survey prevalence in young animals was similar to older animals consistent with previous reports in Costa Rica (Pérez et al., 1994; Herrero et al., 1998) and Brazil (Barros et al., 2005). Young animals probably become infected early, develop immunity and serve as reservoirs of infection for other animals (Herrero et al., 1998).

Our study did reveal a slightly higher prevalence of infection in imported cattle compared with cross bred and autochthones breeds. Although not significantly different in this study, it is in agreement with other studies (Knowles et al., 1982; Sahibi et al., 1998b). These results suggest the necessity for further study to investigate the effect of geographic distribution of vector ticks, farm management in the surveyed areas and what role may be played by imported cattle.

In this study, nPCR detected a higher number of infected animals than the cELISA. These discrepancies could be explained by differences in the timing of the parasite presence and the antibody responses in the infected animals as well as the stage of infection. During chronic infection antibodies remain in the circulating blood for a longer period even after a decrease in parasitemia below detection even by PCR or after parasite clearance (Herrero et al., 1998). Conversely, samples positive by nPCR and negative by serology most likely represent recent infection prior to the development of antibody.

5. Conclusion

This is the first epidemiologic study investigating the occurrence of *A. marginale* in cattle in North Central Morocco using both molecular and serologic tests. The data provide important information about the incidence of *A. marginale* infection and the use of these techniques will be useful when planning management and control programs for this disease in Morocco.

6. Conflicts of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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