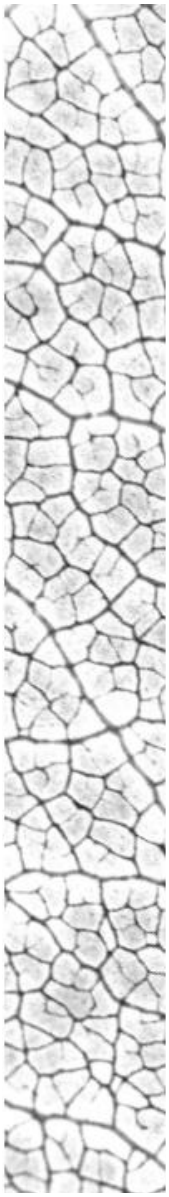

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Molecular detection of anaplasma marginale in ticks naturally feeding on cattle

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Abstract

The present study was carried out to morphological investigation of ticks naturally feeding on cattle, and molecular detection of *A. marginale* in these ticks using the conventional polymerase chain reaction (PCR) assay. Totally 25 infested cattle with ticks were selected from rural areas in Wasit province / Iraq, during March-2020 to June-2020. The findings of clinical observation reported that ticks were distributed onto different bodily parts; udder and testis (50.3%), neck (15.98%), perineal region (28.4%), and ear (5.33%). Although hard (*Ixodid*) tick was the only type detected, two species of different genera were identified among infested cattle; *Hyalomma anatolicum* (75.15%), and *Rhipicephalus sanguineus* (24.85%). Regarding tick species, *H. anatolicum* was more prevalent (P<0.05) than *R. sanguineus* in neck (18.11%) region; whereas, *R. sanguineus* was prevalent significantly (P<0.05) in udder and testis (61.9%) compared to *H. anatolicum*. Regarding bodily regions, *H. anatolicum* and *R. sanguineus* were prevalent significantly (P<0.05) in udder and testis (75.15% and 61.9% respectively). Also, the range and mean number of ticks on each animal was 2-34 and 12.59 respectively. Based on the life stage of collected study ticks, 47.93%, 33.73%, and 18.34% were male, female, and nymph, respectively. Ticks of each study cattle were considered as a one sample and subjected collectively to DNA extraction. Hence, the overall findings for testing 25 samples of ticks by PCR revealed that 4 (16%) of samples were positives for *msp1β* gene specific to *A. marginale*. Additionally, male *Hyalomma anatolicum* ticks were the only positives for *A. marginale* isolates.

Keywords: hard ticks, polymerase chain reaction, Hemoparasites, Tick-borne disease, Iraq

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INTRODUCTION

Anaplasma marginale is one of the most prevalent arthropod-borne hemolytic pathogens, worldwide, which first described by Sir Arnold Theiler in erythrocytes of South African cattle as marginal points (Palmer, 2009; Kocan et al., 2010). Although, *A. marginale* is most correlated to cattle, other animals such as water buffaloes, bisons, African antelopes, and mule deer can infected persistently (Kocan et al., 2003). In cattle, *Anaplasma* parasitizes erythrocytes that subsequently phagocytized by bovine reticuloendothelial cells resulting in fever, mild to severe anemia, icterus, weight loss, lethargy and often death in animals older than 2 years (Yasini et al., 2012).The most survive animals can persistently infected to act as carrier with lifelong immunity, and can serve as reservoir for the pathogen through provision a source of infective blood for both biological and mechanical transmission by ticks (Aubry and Geale, 2011).

Globally, there are approximately 20 species of ticks incriminated in biological transmission of *A. marginale* mostly from stage to stage “transstadial” or within the stage “intrastadial” and rarely through transovarial transmission (Rajput et al., 2005). However, the development cycle of *Anaplasma* is complex and coordinated with the tick feeding cycle (Blouin and Kocan, 1998). Within tick, organisms existed into the parasitized erythrocytes develop in gut cells by binary fission to form large colonies of dense form of organisms that infect many other tick tissues such as salivary gland (Kocan et al., 1992; Ribeiro and Lima, 1996). During tick feeding via salivary gland, cattle become infected with the dense form that having the ability for survival outside the host cells (Kocan et al., 2002; de la Fuente et al., 2007).

In animals, many diagnostic techniques are available for detection *A. marginale* in blood such as direct microscopic detection of *Anaplasma* inclusions in Giemsa-stained blood smears, in addition to several

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serological molecular assays (Nair et al., 2013). In ticks, molecular detection targeting specific genes of *A. marginale* offers the gold standard diagnostic tool in several cross-sectional epidemiological studies because of its high sensitivity and specificity (Torina et al., 2012; Ybanez et al., 2012). The *Msp5*, a highly conserved 19-kDa protein, is encoded by a single-copy gene on the genome of *Anaplasma*. Polymerase chain reaction (PCR) method based on *Msp5* has been previously used to detect low levels of pathogens in cattle experimentally infected cattle with *A. marginale* (de Echaide et al. 2001; Ybanez et al. 2012). In Iraq, rare data are available concerned to the role of ticks in transmission of different infections. Therefore, the present study was aimed to morphological investigation of ticks naturally feeding on cattle, and molecular detection of *A. marginale* in these ticks using the conventional PCR assay.

MATERIALS AND METHODS

Ethical approval

This study has performed following the criteria of the College of Medicine-Wasit University. The collection of tick samples was approved by the Scientific and Ethical Committee of the College of Veterinary Medicine, Wasit University, Wasit / Iraq.

Samples and data

An overall 25 infested cattle with ticks of different ages and sex were selected for the present study from a number of rural areas in Wasit province / Iraq, during March-2020 to June-2020. Ticks of each animal distributing at different bodily parts were collected manually using the surgical forceps by rotating manner and kept into glass container. All tick samples of all infested animals were transported cooled to the laboratory to be subjected for DNA extraction. Data concerned bodily distribution of ticks and the total numbers on each study cattle were reported. Additionally, genus and species of collected ticks and their life stages were investigated in the present study. Morphological identification of collected ticks was based on the characteristics of each genus and species (Janbakhsh, 1957; Walker, 2003).

Molecular testing

DNA Extraction: According to manufacturer's instruction of G-spin™ Total DNA Extraction Kit (Intron Biotechnology, Korea), ticks of each animal were considered as a solely sample to be subjected for DNA extraction following the Type H Protocol. The purity and concentration of all extracted DNA's samples were measured respectively at 260nm and 50µg/ml using of Nanodrop system (Thermo-Scientific, UK). The eppendorf tubes of extracted DNAs were kept frozen at -20°C.

DNA Amplification: Targeting *msp1β* gene, one set of primers [Am.F: 5´- ATGACAGAAGAC GACAAGCAAC -3´) and (Am.R: 5´- AGTAACAATTGCTTGGTCGT - 3´) was used to detect *A. marginale* at 1900bp amplicon size (Molad et al.,-2006). Following the protocol described by Accupower® PCR Premix Kit (Bioneer, Korea), master-mix of extracted DNA samples was prepared at 20µl final volume. Amplification of DNA samples was performed in Thermal Cycler system (Bio Rad, USA) following these conditions: 1 cycle initial denaturation (95°C/2 min); 35 cycles of denaturation (95°C/30 sec), annealing (56°C/30 sec), and extension (72°C/190 sec); final extension (72°C/5 min), and hold (4°C/forever). The samples of mastermix were kept frozen at -20°C until be analyzed by electrophoresis.

PCR Reaction: Using 1% agarose gel stained with Ethidium bromide, 10µl of each mastermix sample in addition to 5µl of Ladder marker (100-3000bp) were analyzed by electrophoresis at 100V and 80mA for 1 hour, and then visualized under UV trans-illuminator (Clinx Science, China).

Statistical analysis

Data of our study were analyzed by GraphPad Prism (6.01) using the chi-square (x^2) test and the Analysis of Variance test (*ANOVA*). Differences between the study values were considered significant at $P<0.05$.

RESULTS

In totally 25 infested cattle with ticks, findings of clinical observation reported that ticks were distributed on the study animals at different bodily parts as following: udder and testis (50.3%), neck (15.98%), perineal region (28.4%), and ear (5.33%). Although hard (*Ixodid*) tick was the only type detected, two species of different genera were identified among infested cattle; *Hyalomma anatolicum* (75.15%), and *Rhipicephalus sanguineus* (24.85%).

Regarding tick species, statistical analysis for distribution of each one revealed that *H. anatolicum* was more prevalent ($P<0.05$) than *R. sanguineus* in neck (18.11%) region; whereas, *R. sanguineus* was prevalent significantly ($P<0.05$) in udder and testis (61.9%) compared to *H. anatolicum*. However, there were insignificant differences ($P>0.05$) in distribution of both species in perineal region (29.13% and 26.19%) and ear (6.3% and 2.38%) respectively.

In comparison between infested bodily regions, the results showed that *H. anatolicum* and *R. sanguineus* were prevalent significantly ($P<0.05$) in udder and testis (75.15% and 61.9% respectively) in comparison to other bodily regions (**Table 1**). Also, the range and mean number of ticks on each animal was 2-34 and 12.59 respectively. Based on their life stage, 81/169 (47.93%), 57/169 (33.73%), and 31/169 (18.34%) of collected ticks were male, female, and nymph, respectively (**Fig. 1**).

Table 1. Ticks and their distribution on bodily parts of study cattle

Ticks		Total	Bodily region of study infested cattle				*P-value*
Genus	Species	No.	Udder and testis	Neck	Perineal region	Ear	
Hyalomma	*H. anatolicum*	127 (75.15%)*	59 (46.46%)	23 (18.11%)*	37 (29.13%)	8 (6.3%)	0.015
Rhipicephalus	*R. sanguineus*	42 (24.85%)	26 (61.9%)*	4 (9.52%)	11 (26.19%)	1 (2.38%)	0.033
Total		169	85 (50.3%)	27 (15.98%)	48 (28.4%)	9 (5.33%)	0.045

Significance * (P<0.05)

Table 2. Total results of PCR assay

Test	Total No.	Positives	Negatives
PCR	**25**	**4 (16%)**	**21 (84%)**

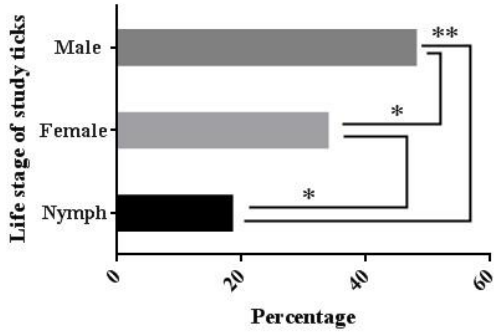

Fig. 1. Life stages for 169 ticks collected from different bodily regions of infested cattle

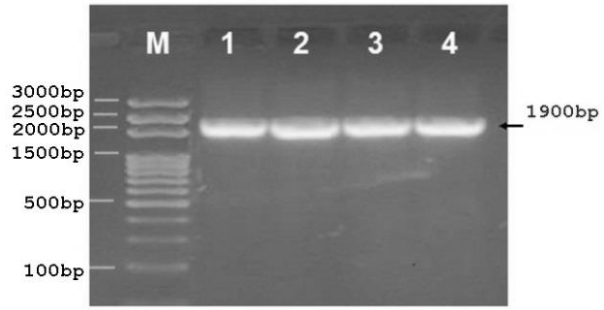

Fig. 2. Gel electrophoresis for PCR products of tick samples targeting *msp1β* gene. Lane (M) represents ladder marker at 100-3000bp; while, lanes 1-4 represent positive samples at 1900bp an amplicon size

Ticks of each study cattle were considered as a one sample and subjected collectively to DNA extraction. Hence, overall findings for testing 25 samples of ticks revealed that 4 (16%) of samples were positives for *msp1β* gene specific to *A. marginale* and 84% of samples were negative (**Table 2**, **Fig. 2**). Additionally, male *Hyalomma anatolicum* was the only positives for *A. marginale* isolates.

DISCUSSION

Ticks are the most common arthropod vector of disease that capable for transmitting a greatest and diverse array of infectious agents to humans and domestic animals. The worldwide emergence or re-emergence of tick-borne diseases is becoming increasingly problematic; however, knowledge of pathogen transmission by ticks is incomplete (Liu and Bonnet, 2014; Wikel, 2018). In Iraq, number studies have performed to detect the role of ticks in transmission of some pathogens in cattle such as *Enterobacteriaceae* (Khalaf et al. 2018), *Theileria* spp. (Al-Fatlawi and Al-Fatlawi, 2019), and *Babesia* spp. (Al-Abedi and Al-Amery, 2020); however, there no reports have performed to detect the role of ticks in carrying of *A. marginale*.

In this study, data of clinical observation showed that ticks were found significantly on udder and testis of cattle livestock. These findings that similar to previous reports (Ndhlova et al. 2009; Hasson, 2012; Monfared et al. 2015) might be attributed to that these regions are near enough for ticks to adhere, thinner skin, in addition to high humidity and ambient temperature which provide suitable protective conditions and allow for blood feeding, egg ling, and hatching of ticks (Tessema and Gashaw, 2010; Sophia et al. 2012; Jonsson et al. 2014).

Based on our results, only two species of ticks were detected, *Hyalomma anatolicum* and *Rhipicephalus sanguineus* with significant prevalence of the first species of tick. These findings were in agreement with that confirmed by local (Al-Abedi and Al-Amery, 2020) and global (Tajedin et al. 2016; Rehman et al. 2017) reports and in contrast with that detected previously (Moghaddam et al. 2014; da Silva et al. 2015). This variation might be governed by the fact that the dynamics of ticks are affected by both the host and habitat environment. In a number of studies, it showed that the effect of host characteristics has conferred various degrees of resistance to tick infestation (Berman, 2011; Carvalho et al. 2011). While, many environmental risk factors have related to infestation of ticks in farm animals, which in turn has a direct impact on the epidemiology of tick-borne diseases such as climate and habitat type (Sajid et al. 2011; Regasa et al. 2015). Unfortunately, the spatial distribution patterns of hard ticks and the ecological mechanisms underlying these patterns are poorly understood and severely limited by the lack of systemic sampling design (Eisen et al. 2014). Therefore, identification of these risk factors could contribute a vital role in designing cost-effective tick control measures.

In the current study, relative high number of ticks per each animal may be explained by the absence of the control tick measures in the study regions and the housing type. In the traditional rural housing, the

prevalence of tick could be increased 13 times as high as the farms with open housing system (Iqbal et al. 2013; Rehman et al. 2017). Age, sex, species, and breed of the animal may represent additional factors for acquisition of ticks and increase the intensity of infestation (Rehman et al. 2017; Vimonish et al. 2020). Also, we showed that the number of male ticks was significantly more prevalent than females and nymphs as reported by Banafshi et al. (2018) and in contrast to findings of Moghaddam et al. (2014). The role of male tick in pathogen epidemiology has not been overlooked because they bite and blood feeding, and may act as reservoirs for transmission of pathogen to females during meeting.

Anaplasma marginale, an obligate intracellular pathogen in the family Anaplasmataceae, is one of the most prevalent blood parasites, worldwide, which confirmed to be responsible on highly economic losses in cattle livestock (Kocan et al. 2010). To date, great numbers of geographical *A. marginale* isolates were found to be varied in their infectivity for ticks, antigenic characteristics virulence, and genotype (Battilani et al. 2017). Characterization of the genetic diversity has performed based on the variability of tandem repeat amino acid sequence located in the N-terminal region of the major surface protein (*Msp*) *1a*, and numerous geographical Msp1a tandem repeats and genotypes were identified (Quiroz-Castañeda et al. 2016). In this study, the MAR1bB2 primer set that designed to specifically amplify the conserved region within the msp1β gene was successfully detected *A. marginale* isolates from ticks as reported previously (Bilgiç et al. 2013). Furthermore, several previously performed reports have indicated that *msp1β* gene in the *A. marginale* genome is a sensitive and specific target for detection of infection both in cattle (Molad et al., 2006; Carelli et al., 2007) as well as in ticks (Goff et al. 1988; Yu et al. 2017). Nonetheless, while *A. marginale* PCR positive ticks were recorded, this does not indicate vector competence; only that the ticks may contain a blood meal from an infected host. Therefore, future studies should evaluate the presence of *A. marginale* isolates in different geographic regions targeting many genes in large and small domestic animals.

CONCLUSION

To our knowledge, the present study is the first report from Iraq which detects *A. marginale* in ticks naturally feeding on cattle using the molecular PCR assay. The MAR1Bb2 primer may be suitable to detect *A. marginale* isolates from various parts of the world; however, sensitivity and specificity of this primer should be tested using a larger panel of isolates. Consequently, research on molecular interactions between ticks and pathogens as well as the identification of suitable antigenic targets is a major challenge for the implementation of new tick-borne diseases control strategies.

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