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Polymerase Chain Reaction Study For Anaplasma Phagocytophilum In Iraqi Cows

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ABSTRACT

The current research was performed in three provinces (Al-Najaf Al-Ashraf, Babylon and AL-Qadisiya) in Iraq to investigate the prevalence of Anaplasma phagocytophilum in cattle. Surveillance was carried out from February to October 2020. A total of 200 of blood samples were randomly collected from cattle from three governorates. The samples were tested for finding of Anaplasma phagocytophilum infection using polymerase chain reaction (PCR) technique using a specific primer(EE1f, and EE2r and nested primers: EE3f, EE4r) and subsequent sequencing of positive samples. The results showed the infection rates of Anaplasma phagocytophilum 15/200 (7.5%).The higher infection rate were 11.4% (80/70) in AL-Qadisiyah and 6.15 %(4/65) in Al-Najaf Al-Ashraf .Whereas the lower infection rate was 4.61% (3/65) in Babylon samples. The highest infection rate months (May, June and July): 3/29 (10.34%), 4/37 (10.18 %), and 4/34 (11.76%), respectively of infection with Anaplasma phagocytophilum. while the lower infection rate months was 0% in February, March, and October. The higher infection rate was recorded in animals aged more than to three years 9.72% (7/72), from two to three year was 7.40% (6/81) and while lower infection rate 4.68% (3/64) was recorded in animals aged less than 2 year. The rate of infection in females was 8.82% (12/136) more than in males 4.68% (3/64).

Keywords: Anaplasma phagocytophilum, PCR, cattle

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INTRODUCTION

A.phagocytophilum is a obligate intracellular, Gram-negative, transmitted by tick , the bacterium replicates in neutrophils (Ismail, 2010). In sheep and cattle, it was initially recognized as the causative agent of tick-borne fever (TBF). (Gordon,1932; Hudson,1950). *A. Phagocytophilum* is known to be a new emerging generic congregation bacterium and is gradually found in Ticks have different hosts in many parts of the world, including domestic animals and humans. (Pazhoom,2016;Dondi *et al.*, 2014;Khatat , 2015). Hyalomma is It is a common genus that affects cows in the southern region of Iraq,; but, H. marginatum turanicum, H. asiatcum ,H. anatolicum anatolicum, H. anatolicum, Rhipicephalus turanicus and Boophilus annulatus excavatum It affects livestock to a lesser extent (Awad, 2006;Mallah, 2016).

Granulocytic anaplasmosis in humans, horses, dogs and cats and tick-borne fever (TBF) in ruminants are caused by *anaplasma phagocytophilum* (Woldehiwet, 2006). Typical clinical signs of TBF include inappetence ,fever, lethargy, sudden decrease in milk production, oedema , lower limb;typical laboratory observations are thrombocytopenia and leukopenia (Pusterla,1997;Tuomi, 1967). Tick borne fever reasons significant decline in milk production causes economic losses and is thought to be underdiagnosed in cattle. (Pusterla, 1997,Pusterla,1998;Nieder *et al.*, 2012).

Most of the studies often concentrated accuracy of PCR tests and can find small quantities of nucleic acid and specific fragments of nucleic acid to differentiate closely related strains. (Dumler,2004;Massung,2003). However, substantial variance within A. *phagocytophilum* strains has been distinguished, and geographic locations or isolates from several hosts have displayed genetic diversity and deviation within often used PCR-target genes(Morissette *et al.*, 2009;Smrdel *et al.*, 2015). The aim of this research was to decide the incidence of *Anaplasmosis* cows in Iraq governorates (AL-Qadisiyah, Al-Najaf Al-Ashraf, Babylon)..,to describe and perform the *16S rRNA* gene sequence modifications and perform their phylogenetic examination.

MATERIALS AND METHOD

Collecting data and experimental animals

In current study, approximately 200 samples from cattle were used in. The sex of these animals was mixture plus their ages were showed from >1 year-old or <3 year-old. The cattle were selected randomly from 3 different regiens of Iraq, which allocated at AL-Qadisiyah, Al-Najaf Al-Ashraf, Babylon;

Collecting samples of blood

The samples of Blood were obtained from the vein (jugular) of cattle with EDTA-vacationer tube. Cooling boxes used to move all samples. The test center applied in College of Veterinary Medicine. /Al-Qadisiyah University

DNA extraction

The *Anaplasma phagocytophilum* genomic DNA from cattle blood samples was extracted using a mini kit of DNA extraction. (Taiwan, Geneaid Biotech Ltd). The genomic DNA *Anaplasma phagocytophilum* extracted tested the 15–60 ng/µl concentration variety of depending on guidelines from the company. A Nano-drop spectrophotometer (260/280 nm). used to analyze the purity of extracted DNA .

The method of PCR

depending upon technique defined the Yang *et al* (2016), and the detection of *Anaplasma phagocytophilum* in blood samples was measured. Versions of extracted DNA (from cattle blood) have been tested using PCR for the presence of *Anaplasma phagocytophilum*; specific gene primers

were designed at approximately 928 bp, as the product size.

First round:

EE1: TCCTGGCTCAGAACGAACGCTGGCGGC

EE2: AGTCACTGACCCAACCTTAAATGGCTG

Second round:

EE3: GTCGAACGGATTATTCTTTATAGCTTG

EE4: CCCTTCCGTTAAGAAGGATCTAATCTC

DNA sequencing

To determine the primers specificity of the blood *Anaplasmosis* in, the cattle identification was confirmed that the positive PCR (928-bp band) products belong to the genome of *Anaplasma phagocytophilum.* Using Sanger's sequencing technique from Macrogen (Korea), the products of two positive PCRs were sequenced. The Maximum Compound Likelihood method were applied to evolution of spaces that were calculated by using phylogenetic tree UPGMA (MEGA 10 version).

Statistical analysis

In current study, SPSS- software were applied to analyze the data. To determine the relationship between the variable outcomes, a Chi-square test was used.

RESULTS

Identification of DNA Anaplasmosis

The PCR findings demonstrated that 15 out of 200 samples (7.5%) positive to *Anaplasma phagocytophilum* (Fig.1, 2). Moreover, the incidence was greater in AL-Qadisiyah thanAl-Najaf Al-Ashraf (11.4% (8/70) and 6.15% (4/65), respectively) compared to that in Babylon region (4.61% (3/65) (Table 1 and Fig.1, 2).

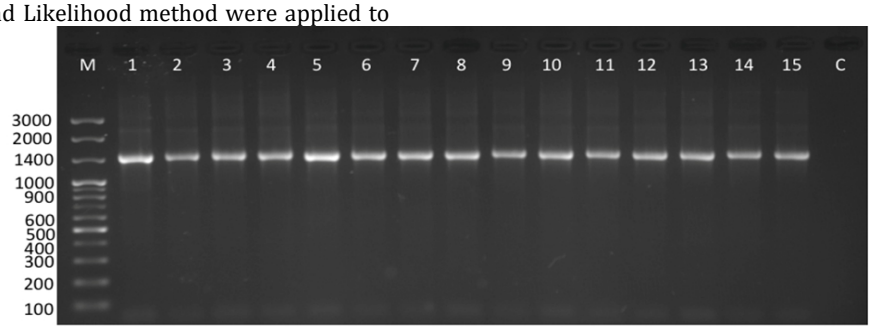

Figure 1: Gel electrophoresis image (1 % agarose) shows the PCR products of *Anaplasma phagocytophiulm* using E1 and E2 (first round PCR). (1-15 are positive samples while C is control negative in which H2O was used instead of DNA). M is molecular ladder (AddBIO, Korea).

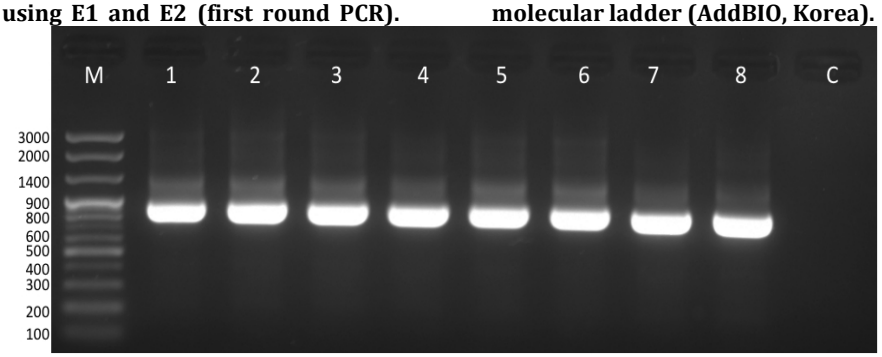

Figure 2: Gel electrophoresis image (1 % agarose) shows the PCR products of *Anaplasma phagocytophiulm* using EE-3 and EE-4 (second round PCR). (1-8 are positive samples while C is control negative in which H2O was used instead of template DNA). M is molecular ladder (AddBIO, Korea).

Table (1) Infection Rate with *Anaplasma phagocytophilum* by PCR according to regions of Study:.

Governorate	No. of examined cattle	No. of infected cattle with *A.phagocytophlium*	Percentage (%)
AL-Qadisiyah	70	8	11.4
Al-Najaf Al-Ashraf	65	4	6.15
Babylon	65	3	4.61
Total	200	15	7.5

X2: chi-square value (X2 = **2.507(NS)**.

These outcomes also illustrated that the incidence of *Anaplasma phagocytophilum* was non-significant by cattle gender (Table 2). However, the infection rate was higher in females (8.82%) compared to male (4.68%) (Table 2). Interestingly, the results demonstrated that the higher rate of infection was measured in aged animals (above 3 years) matching of 9.72% (Table 3). Conversely, the lower rate of the infection was registered in less than 2 years (4.25%, Table 3).

Table (2) Infection rate with *Anaplasma phagocytophilum* by PCR examination of blood according to sex of cattle

Sex	No. of tested animals	No. of infected animals	Percentage (%)
Males	64	3	4.68

Females	136	12	8.82
Total	200	15	7.5

X2: chi-square value (X2 =**1.037(NS)**.

Table (3): Infection rate with *A. phagocytophilum* by PCR according to age of groups

Age / year	No. of tested cattle	No. of infected cattle	Percentage (%)
6 months-2 years	47	2	4.25
2-3 years	81	6	7.40
Above 3 years	72	7	9.72
Total	200	15	7.5

X2: chi-square value (X2 =**1.227(NS)** .

Study of seasonal difference the maximum level of *A. phagocytophilum* infection was registered during the Iraq (May, June and July) months of summer 3/29 (10.34%), 4/37 (10.18 %), and 4/34 (11.76%), respectively (Table 4). There were non-significant differences between those months, however. All samples obtained during February, March, and October, on the other hand, were negative for *A. phagocytophilum* (Table 4).

Table (4) incidence of *Anaplasma phagocytophilum* according to the months .

Months	NO. of blood samples	Infected	(%)
February	12	0	0
March	10	0	0
April	23	1	8.69
May	29	3	10.34
June	37	4	10.18
July	34	4	11.76
August	27	2	7.40
September	14	1	7.14
October	14	0	0
Total	200	15	7.5

X2: chi-square value (X2 = **5.066 (NS**).

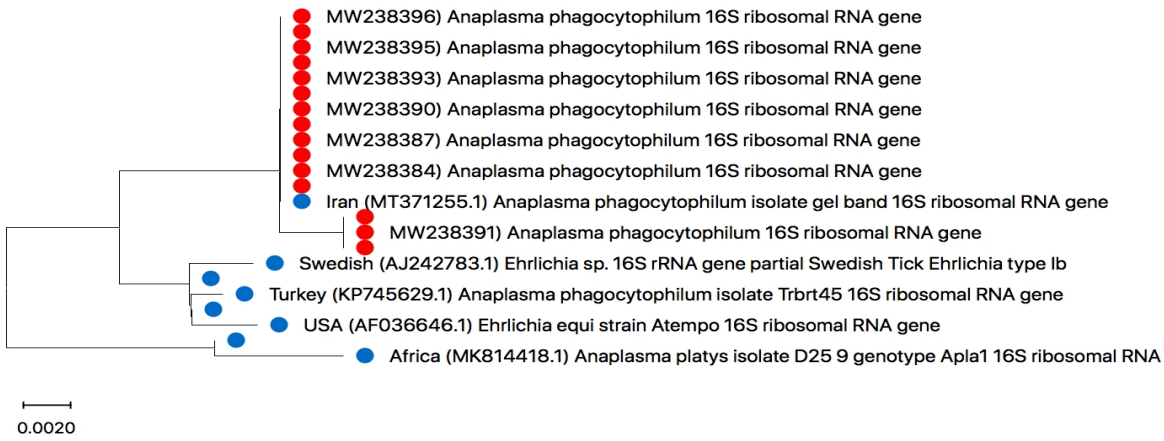

Figure 3: Phylogenetic tree analysis of *Anaplasma phagocytophilum* (16s rRNA) gene of the currently identified sequences referred as blue circles with their corresponding accession numbers (deposited in the NCBI bank gene as following: MW238384, MW238385, MW238386, MW238387, MW238388, MW238389, MW238390, MW238391, MW238392, MW238393, MW238394, MW238395, MW238396, MW238397, and MW238398). As can be seen, the Iraqi strains mainly appeared in two branches that are mostly closely related to the Iranian strain while the other strains appeared slightly different from the first one.

Validation of PCR products by sequencing

According to *Anaplasma phagocytophilum* genomes, the findings of *Anaplasma phagocytophilum* sequencing were analyzed and the banked NCBI GenBank database were used in Basic Local Alignment Search Tool (BLAST).

Anaplasma phagocytophilum phylogenetic analysis

A tree of simple phylogenetic created in basis of the partial sequences of EE1,EE2,EE3,EE4 to study the

relationship between Anaplasma phagocytophilum strains found in the current study and Anaplasma phagocytophilum available in the GenBank database at the NCBI. The partial sequences of EE1,EE2,EE3,EE4 gene of 15 positive *A. phagocytophilum* samples (GenBank accession numbers MW238384, MW238385, MW238386, MW238387, MW238388, MW238389, MW238390, MW238391, MW238392, MW238393, MW238394, MW238395, MW238396, MW238397, and MW238398). They have been shown to be closely linked to anaplasma Phagocytophilum isolates present in GenBank (Fig3).

DISCUSSION

A. phagocytophilum is an intracellular, bacterium that causes tick-borne fever in many species of mammals, including domestic ruminants (TBF) and granulocytic anaplasmosis in humans.(Silaghi *et al*., 2018). *A. phagocytophilum* DNA was identified by nested PCR for the intensification of the 16S rRNA gene as formerly described (Barlough,1996;Ogden,1998;Gokce *et al*., 2008;Yang *et al*., 2013). PCR-based approaches are effective instruments and play a An important role in affirmation of Infection with *Anaplasma phagocytophilum* in specimens of environmental and clinical origin. Frequent PCR amplification analyses and primer sets for recognition of *Anaplasma phagocytophilum* have been identified since the first discovery of the HGA agent in 1994. (De La Fuente *et al*., 2005;Chen,1994). These consistent rates of infection result from what has been observed in previous studies carried out by (Yang *et al.*, 2015).The obtained prevalence of *A.phagocytophilum* (7.5) was lower than that reported in Turkey(Gokce *et al*., 2008), (14.75%) and(Aktas,2015).(30.8%), in China(Zhang *et al*., 2012).(23.38%)and(Yang *et al*., 2013),(35.0 %) . This prevalence, by comparison, was higher than those reported in Pakistan (Iqbal *et al*., 2019), (2.66%), Iran(Noaman & Shayan, 2009),(1.33%). In reality, infection rates between countries have been reported to be variable and may even differ significantly between neighboring farms. (Stuen,2002). Infection with *Anaplasma phagocytophilum* has been recorded in domestic animals in China and humans, wild, and incidence in diverse hosts or geographic locations have been variable. (Yang *et al*., 2016). Our findings show that infection rates differ slightly by sex and suggest that female cattle have a higher rate of infection than males. This may be because female are more vulnerable to multiple physiological changes, such as stresses induced by pregnancy, childbirth and lactation. (Belal *et al*, 2014). The age group above 3 years of age reported the highest rates of infection. These results are compatible with (Atif,2013; Chaudhry,2010) Who reported that the highest incidence of adult animal infection may be due to the chronicity of infection. Another review by (Kamani *et al*., 2010) Higher infection rates have been shown to be greater in older age groups. Such observations were also agreed with (M'Ghirbi *et al*., 2016). This variance could be detected by the significant exposure of adults to the tick vectors; Calves are provisionally protected (maternal antibodies) by the colostrum and a mother's immunity, preventing short-term protection. The feeding of each tick vector individually can be distributed seasonality and geographical of infections with *Anaplasma phagocytophilum*, which might likely determine. The cases were observed from April to July were observed in October (Poitout, 2005). Most cases have been reported to occur between April and September, and the seasonal spread of the disease most likely represents peak tick activity periods. This result is in line with (Beall *et al*., 2008).

Indicative of that several tick species may sustain or be involved in the transmission of *Anaplasma phagocytophilum*. As a number of tick species are considered to be host specific (Wells *et a*l., 2013), We believe that sheep have been infested with tick vectors that feed preferentially on sheep compared to cattle, resulting in a higher prevalence of *Anaplasma phagocytophilum* in sheep .These finding show that both sheep and cattle are part of the natural maintenance cycle of *Anaplasma phagocytophilum. Anaplasma phagocytophilum* seems to exhibit ecotypes with diverse host ranges and zoonotic potential (Jahfari *et al*., 2014).

(Chaudhry *et al*., 2010) A high was recorded in the months of June-August, when the seasonal prevalence of vector ticks very high transmitting infection from carrier animals to other healthy animals in the herd who indicate that the seasonal dynamics of tick activity was the greater in April, May, June, and July when associated with the highest prevalence of *Anaplasma phagocytophilum* infection.The findings of the current study confirm the presence of *Anaplasma Phagocytophilum* in central Iraq and the potential risk of transmitting this infection to humans .To control the *anaplasma phagocytophilum* we still have to identify the transmitting vectors, animal reservoirs and pathogenesis of *Anaplasma phagocytophilum* in human and animals in Iraq. is important to the improvement of control strategies for *Anaplasma phagocytophilum* in Iraq.

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