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SEROLOGICAL DETECTION OF *COXIELLA BURNETII* CHRONIC INFECTION-PHASE 1 IN SERUM OF HUMAN AND SHEEPAT AL-QADISIYAH PROVINCE, IRAQ

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ABSTRACT:

The present study was carried out to estimate the prevalence of chronic infections with *C. burnetii* among humans and sheep sera using of specific-species enzyme-linked immunosorbent assay (ELISA). At different regions in Al-Qadisiyah province/Iraq, an overall 366 blood samples that involving 182 human and 184 sheep were collected aseptically during the period of February to November, 2018. Total prevalence of *C. burnetii* phase-1 was 7.38% sero-positive samples that comprising 3.3% humans and 11.41% sheep. Regarding to residence factor, significant elevation ($P<0.05$) in sero-positive results among rural areas were recorded in both human (7.94%) and sheep (15%) samples. Data concerning to age factor were detected on significant increases ($P<0.05$) in sero-positive results of humans having an age of >30 years (3.42%), and in sheep having an age of >24 months (27.08%). Among sex factor, significant increases ($P<0.05$) in sero-positive prevalence of chronic infections were seen in samples of male human (6.56%) and female sheep (12.84%).

KEYWORDS: *Coxiella burnetii*, Serum, Human, Sheep, Chronic infection, Al-Qadisiyah, Iraq.

1. INTRODUCTION

Coxiella burnetii is one of the most important gram negative intracellular bacteria related to Coxiellaceae family of Legionellales order. Although the genus of *Coxiella* is similar morphologically to Rickettsia; a variety of genetic and physiological differences were detected recently (Chmielewski and Tylewska - Wierzbanowska, 2012; González-Quijada et al., 2015). This pathogen causes a zoonotic infection Q fever (coxiellosis) that having a worldwide distribution. According to the Centre for Disease Control and Prevention (CDC), *C. burnetii* is recognized as a potential agent of bioterrorism and classified under the category of (B) agents (Azad and Radulovic, 2003; Anastacio et al., 2013). *Coxiella* can exit in environment for weeks and transport for long distance by wind (Hilbert et al., 2012). Also, the bacteria can be found at massive number in fetal membranes, placenta, amniotic fluid of aborted infected animals in addition feces and urine of infected animals (Berri et al., 2000; Agerholm, 2013). In human, it thought that aerosol route is the primary route of infection. However, farm animals particularly sheep are the most implicated ones as they act as reservoir for pathogen (Angelakis and Raoult, 2010; Georgiev et al., 2013).

In most human *C. burnetii*, infections are manifested by a clinical polymorphism and frequent asymptomatic disease ranged from acute mild self-limiting illness to chronic severe illness (Kazar, 2005). However, the clinical symptoms of acute disease may include fever, cough, headache, chronic fatigue, pneumonia, hepatitis and myocarditis, whereas the chronic form may develop endocarditis and vascular infections (Terheggen and Leggat, 2007). In sheep, disease syndrome is relatively less occurrence and characterized by reproductive disorders that impact the production and economic efficiency of the farm (Berri et al., 2002).

Recently, many laboratory diagnostic approaches are available for detection of humans and animals coxiellosis including the culture, molecular and serological tests using various samples (Merhej et al., 2013). Nowadays, polymerase chain reaction (PCR) is considered as the most ideally techniques that applied for almost early acute cases have not mounted an antibody response yet during the incubation period (2-4 weeks) (Rolain et al., 2005; Wegdam-Blans et al., 2012). In routine practice, laboratory diagnosis is based mainly on serology as the majority of samples will not be submitted to the laboratory with 2 weeks after the onset of the infection (Wegdam-Blans et al., 2012). Complement fixation test (CFT),

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indirect fluorescent antibody test (IFAT) and the enzyme-linked immunosorbent assay (ELISA) are most available effective serological techniques for diagnosing of *C. burnetii* infections (Slaba et al., 2005; Anderson et al., 2013). Regarding the technical considerations, ELISA seems to be more appropriate in routine diagnosis and large scale epidemiological studies that aimed at demonstrating the exposure to *C. burnetii* (Guatteo et al., 2007; Porter et al., 2011).

Unfortunately, very few studies have been published on the prevalence of infection in Iraq, especially in Al-Qadisiyah province. Hence, the present study was designed to estimate the sero-prevalence of chronic infections with *C. burnetii* phase-1 among humans and sheep sera by species-specific an indirect ELISA. In addition, the association of ser-positivity to residence, age and sex that considered as epidemiological risk factors were also detected among both human and sheep samples.

MATERIALS AND METHODS

Samples

At different regions in Al-Qadisiyah province / Iraq, an overall 366 samples (182 humans and 184 sheep) were selected randomly to present study, during the period of February to November, 2018. Blood were the main specimen that drained aseptically using disposable an EDTA vacutainers from a superficial vein in the hind limb of humans, and from jugular vein of sheep. All sera were placed into 1.5 ml numbered eppendorf tubes that saved frozen at $^{-}20^{o}$C until tested by ELISA. Residence, age and sex of study samples (humans and sheep) were considered as epidemiological risk factors.

Serology

Based on the manufacturer's instructions, species-specific an indirect ELISA kits (*MyBioSource, USA*) were used in this study for qualitative immunoenzymatic determination of *C. burnetii* phase-1. Briefly, the ELISA was performed as following:

For human: All sera samples were thawed, mixed by vortex, and diluted by dispensing 10 µl of serum sample and 1ml of IgG of Sample Diluent into eppendorf tubes to obtain a 1+100 dilution that mix thoroughly by a vortex. The first five wells in each ELISA kit were used for blank (1 well), negative control (1), cut off control (2 wells), and positive control (1 well), while other wells were allocated for diluted samples, which dispensed with 100 µl from each one except the blank. Then, the micro-plate was covered with foil, incubated at 37^{o}C for 1 hour, and washed three times with 300 µl of Washing Solution for each well after removing of foil. A100µl of Conjugate was added to each well except the blank, covered with foil, incubated for 30 minutes at room temperature, and washed three times with 300 µl of Washing Solution for each well after removing of foil. A 100µl of TMB Substrate Solution was added to each well, incubated exactly for 15 minutes at room temperature, dispensed with 100µl of Stop Solution, and then the optical density (OD) was measured at 450 nm.

For sheep: All sera were thawed at room temperature. From each kit, two positive two negative wells were pipetted with 50µl of positive and negative control for each well. A 10 µl of serum sample in addition to 40µl of Sample Diluent was added to each sample well. A 100µl of HRP-conjugate reagent was added to all wells, covered with foil, incubated for 60 minutes at 37^{o}C, and washed four times with Washing Solution. A 50µl of Chromogen Solution A and 50µl of Chromogen Solution B were added to each well, mixed gently, and incubated at 37^{o}C for 15 minutes. A 50 µl of Stop Solution was added to each well, and the optical density was read at 450 nm.

Statistical analysis

All obtained results were introduced, tabled, figured, and then analyzed using of two computerized programs, Microsoft Office Excel (V_{2013}) and IBMLSPSS (V_{23}). Statistical descriptive analysis, Chi-square test (X^2) and *t-test* were applied. Statistical differences were considered significant at a level of $P<0.05$ (Petrie and Watson, 2006). Statistical associations between the positive results of the epidemiological risk factors were also detected.

RESULTS

- Of 366 serum samples, a total prevalence of sero-positivity was 7.38% that comprising 3.3% human and 11.41% sheep samples. Significant differences ($P<0.05$) were reported among positive results of humans and sheep, (Table 1).

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Table (1): Total results of humans and sheep tested by ELISA

Sample	Positive	Negative	Total
Human	6 (3.3 %) *	176 (96.7 %)	182
Sheep	21 (11.41 %) **	163 (88.59 %)	184
Total	27 (7.38 %)	339 (92.62 %)	366

* Significance ($P<0.05$)

- Regarding to residence factor, there were a significant elevation ($P<0.05$) in sero-positive results among rural areas in (7.94%) of human (Table 2), and in (15%) of sheep samples (Table 3).

Table (2): Results of human samples based on residence factor

Residence	Positive	Negative	Total
Urban	0 (0 %) *	92 (100 %)	92
Sub-urban	1 (3.70 %) *	26 (96.30 %)	27
Rural	5 (7.94 %) **	58 (92.06 %)	63
Total	6 (3.30 %)	171 (93.96 %)	182

* Significance ($P<0.05$)

Table (3): Results of sheep samples based on residence factor

Residence	Positive	Negative	Total
Urban	0 (0 %) *	18 (100 %)	18
Sub-urban	3 (6.52%) **	43 (93.48%)	46
Rural	18 (15%) ***	102 (85%)	120
Total	21 (11.41%)	163 (88.59%)	184

* Significance ($P<0.05$)

- Data concerning to age factor were detected significant increases ($P<0.05$) in sero-positive results of humans having an age of >30 years (3.42%), and in sheep having an age of >24 months (27.08%) as showed in (Table 4) and (Table 5), respectively.

Table (4): Results of human based on age factor

Age/Y	Positive	Negative	Total
< 10	0 (0 %) *	5 (100 %)	5
> 10 - 30	2 (3.33%) **	58 (96.67%)	60
> 30	4 (3.42%) ***	113 (96.58%)	117
Total	6 (3.3 %)	176 (96.7 %)	182

Y (Year), * Significance ($P<0.05$)

Table (5): Results of sheep samples based on age factor

Age/ M	Positive	Negative	Total
≤ 6	0 (0 %)	9 (100 %)	9
> 6-12	0 (0 %)	35 (100 %)	35
> 12-24	8 (8.7%) *	84 (91.3%)	92
> 24	13 (27.08%) **	35 (72.92%)	48
Total	21 (11.41 %)	163 (88.59 %)	184

M (Month), * Significance ($P<0.05$)

- Among sex factor, significant increases ($P<0.05$) in sero-prevalence were recorded in male humans (6.56%), (Table 6), and female sheep (12.84%), (Table 7).

Table (6): Results of human samples based on sex factor

Sex	Positive	Negative	Total
Female	2 (1.65%) *	119 (98.35)	121
Male	4 (6.56%) **	57 (93.44%)	61
Total	6 (3.3 %)	176 (96.7 %)	182

* Significance ($P<0.05$)

Table (7): Results of sheep samples based on sex factor

Sample	Positive	Negative	Total
Female	19 (12.84%) **	129 (87.16%)	148
Male	2 (5.56%) *	34 (94.44%)	36
Total	21 (11.41 %)	163 (88.59 %)	184

* Significance ($P<0.05$)

DISCUSSION

To control *C. burnetii* transmission among animals and from animals to humans, it is vital to have tools that reliably detect the pathogen shedders. In this study, the total prevalence of *C. burnetii* pahse-1 among all study samples was 7.38% involving 3.3% and 11.41% sero-positive humans and sheep, respectively. However, these results were lower than detected in human (31.5%) by (Hafeth et al., 2010) and higher than seen in sheep (5.8%) by (Abed et al., 2010). Worldwide, sero-prevalence of *C. burnetii* in human was 5.2% in Australia (Tozer et al., 2011), 11% in Denmark (Bosnjak et al., 2010), 15.3% in Spain (Cardenosa et al., 2006), 18% in Netherlands (Blaauw et al., 2012), 19.8% in Iran (Mobarez et al., 2017), 12.3-32 in Turkey (Kilic et al., 2008; Gozalan et al., 2010), and 35.8% in Kenya (Njeru et al., 2016); whereas in sheep, there was 9% in Italy (Masala et al., 2004), 17% in USA (McQuiston and Childs, 2002), 18% in Kenya (Knobel et al., 2013), 20% in Iran (Asadi et al., 2013), and 20% in Turkey (Kennerman et al., 2010). Variations in *C. burnetii* prevalence among this study might be attributed mainly to diagnostic technique, type and method of sample collection, and therapeutic approach. In most studies, it seemed that *C. burnetii* is more incidence in sheep than humans, which might be attributed to the fact that sheep act as reservoir for the pathogen as well as they act as a major source for human infections (Roest et al., 2013; Contreras et al., 2018). In last decades, several large human outbreaks were attributed to sheep. Shedding of *C. burnetii* during parturition

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can occur in milk, feces, birth fluids in addition to placental membranes, which become aerosolized to be transmitted for human by inhalation (Van den Brom et al., 2015).

The association of residence to *C. burnetii* phase-1 infections was also discussed in both humans and sheep. In this study, the results demonstrated that the rural areas having higher sero-positives comparing to sub-urban and urban areas, respectively, as detected by many studies (Mediannikov et al., 2010; Knobel et al., 2013; Angelakis et al., 2014). These findings could be attributed to that the people in rural areas were most frequently to direct/indirect contacts with infected farm ruminant that act as active reservoirs and shedders for *C. burnetii* to their environment. Many studies hypothesized that the percent of humans and animals was having an association with the sero-positivity as up to 1×10^9 bacteria can shed in placenta of infected sheep/goats that linked to human cases of Q-fever (Sánchez et al., 2006; Meadows et al., 2017).

The relationship of sero-positive results between different age groups was detected in this study as the increase ages were associated with elevation of sero-positivity particularly at >30 years of age in humans and >24 months of age in sheep. In human, epidemiological data clearly showed that there were increases in incidence and severity of symptomatic infections. Indeed, the relative risk of *C. burnetii* reported that the adolescents are five times more symptomatic following infection than younger children; and the adults of 60-69 year age range is five times higher than the adults of around 40 years of age (Maltezou and Raoult, 2002; Raoult et al., 2005). Although, many reasons are suggested to clarify the age-increased susceptibility for bacterial infection such as a dysfunction of immune system as well as the age-associated physiological and anatomical changes, the actual causes remain unclear (Leone et al., 2007). However, (McCaughey et al., 2008) suggested that most people acquired *C. burnetii* infection between ages of 25 to 34 and, after that, age sero-prevalence remained stable. In sheep, older animals showed high sero-positive rates to *C. burnetii* infection, as reported by many studies (Anastacio et al., 2013; Souza et al., 2018). This higher sero-prevalence was expected due to the more probability of repeated contacts with the pathogen proportionally to lifespan (Rizzo et al., 2016).

In sex factor, the results of present study in humans reported that males are more sero-positivity than females; whereas in sheep, sero-prevalence of *C. burnetii* IgG antibodies was higher in females than males. In humans, the obtained results were agreement to that detected by (Leone et al., 2004) who hypothesized the role of sex hormone in pathogenesis of *C. burnetii* infection, and with (Dupont et al., 1992) who found that men having 2.5 times more likely to be infected with the pathogen than women. Indeed, the results of these epidemiological studies suggested that sex hormones may be involved in control of expression of clinical presentation of *C. burnetii* infection. In sheep, the high sero-positive infections in females were corporate with (Meadows et al., 2015) and conflicted with (Asadi et al., 2014) who recorded that there were no differences between females and males. This might be explained by the facts that females are more animals than males, exposed to more physiological exercise than males during pregnancy and milk production, and received less attention than males. In most farms, rams of good characteristics that selected for reproduction, were not allowed to roam freely in the pastures either to maintain on them from infection, to avoid their aggressiveness or to prevent them from mating with ewes at estrus (Souza et al., 2018).

In conclusion, results of present study revealed a relatively high sero-prevalence of *C. burnetii* phase-1chronic infections in both samples, particularly sheep, at Al-Qadisiyah province. The residence, age, and sex were the significant epidemiological risk factors for pathogen infections. Further studies are essential to confirm the actual role of farm animals, at different regions, in sharing of pathogen to humans.

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APPENDIXES

Appendix (1): Manufacturer's validation of human ELISA kit

Substrate blank (A_1)	OD < 0.100
Negative control (B_1)	OD < 200 and < Cut-Off
Cut-Off control (C_1 and D_1)	OD > Cut-Off
Positive control (E_1)	OD > Cut-Off control
Positive sample	OD > Cut-Off
Negative sample	OD < Cut-Off

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Appendix (2): Manufacturer's validation of sheep ELISA kit

Average of OD positive controls (PC)	OD ≥ 1.0
Average of OD negative controls (NC)	OD ≥ 0.15
Cut-Off	NC + 0.15
Positive sample	OD > Cut-Off
Negative sample	OD < Cut-Off
Negative sample	OD < Cut-Off

Appendix (3): Interpretation standard results of human ELISA kit

Standard criteria	OD
Substrate blank P	< 0.72
Negative control P	< 183
Cut off	241
Cut off control	318
Positive control	1176
Positive samples	> 241
Negative sample	< 241

Appendix (4): Interpretation standard results of sheep ELISA kit

Standard criteria	OD
Average of OD positive controls (PC)	2.04
Average of OD negative controls (NC)	0.91
Cut-Off	1.06
Positive sample	> 1.06
Negative sample	< 1.06

Appendix (5): Number, titer and OD sample/Cut-off of human sero-positive samples

No.	Titer (OD_{sample})	OD_{sample} / Cut-off
9	287	1.19
48	304	1.26
117	243	1.01
131	291	1.21
142	268	1.11
176	272	1.13
M ± SE	277.5 ± 8.73	1.15 ± 0.04

Mean (M), Standard error (SE)

Appendix (6): Number, titer and OD sample/Cut-off of sheep sero-positive samples

No.	Titer (OD_{sample})	OD_{sample} / Cut-off
13	1.23	1.16
20	1.18	1.11
27	1.54	1.45
41	1.62	1.53
68	1.09	1.03
89	1.26	1.19
91	1.30	1.23
106	1.14	1.08
113	1.38	1.3
114	1.29	1.22
127	1.08	1.02
129	1.51	1.42
140	1.42	1.34
147	1.07	1.01
159	1.37	1.29
160	1.28	1.21
161	1.11	1.05
168	1.16	1.09
172	1.38	1.3
178	1.22	1.15
181	1.40	1.32
R (M ± SE)	1.29 ± 0.034	1.21±0.032

Range (R), Mean (M), Standard error (SE)

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