Research Article

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Cross-sectional Molecular Detection of *Francisella tularensis* in Domestic Rabbits in Sulaimani Province Kurdistan Region, Iraq

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Abstract

Tularemia is one of the diseases transmitted between humans and animals. It is caused by a Gram-negative bacterium *Francisella tularensis.* Recent serological studies suggested that tularemia can be an endemic bacterial zoonotic disease in some countries surrounding Iraq such as Iran and Turkey. The main objective of this study is to detect tularemia in Sulaimani province northeast Iraq near to Iran border. Sulaimani city also has a contact with many Turkish cities. This study was conducted between Jun and October 2020. Blood samples were taken from one hundred local breed rabbits with different ages and sexes. A highly sensitive real time PCR technique was used. Sixteen out of one hundred blood samples (16%) were positive taken from different local breed rabbits from four different places in Sulaimani province. All positive samples detected in the center of Sulaimani city. No published documents have been reported yet about tularemia in Kurdistan Region. This paper documented molecular detection of *F. tularensis* in local breed rabbits in Sulaimani province Kurdistan Region-Iraq.

Keywards: Tularemia, Sulaimani city, zoonotic disease, PCR

الكشف الجزيئي المقطعي عن فرنسيسيلا تولارنسيس في الأرانب الأليفة في محافظة السليمانية ، إقليم كردستان العراق

الخلاصة

التولاريميا هو مرض حيواني المصدر تسببه بكتيريا سالبة الجرام Francisella tularensis. اقترحت الدراسات المصلية الحديثة أن مرض التولاريميا يمكن أن يكون مرضًا جرثوميًا حيوانيًا مستوطنًا في بعض البلدان المحيطة بالعراق مثل إيران وتركيا. الغرض من هذه الدراسة هو الكشف عن مرض التولاريميا في محافظة السليمانية شمال شرق العراق بالقرب من الحدود الإيرانية. كما أن مدينة السليمانية على اتصال بالعديد من المدن التركية. أجريت هذه الدراسة في الفترة ما بين يونيو وأكتوبر 2020. تم أخذ عينات الدم من مائة أرنب من السلالات المحلية من مختلف الأعمار والأجناس. تستخدم تقنية تفاعل البوليميراز المتسلسل في الوقت الحقيقي شديدة الحساسية. ستة عشر من أصل مائة عينة دم (16٪) كانت إيجابية مأخوذة من سلالات محلية مختلفة من الأرانب من أربعة أماكن مختلفة في محافظة السليمانية. تم الكشف عن جميع العينات الإيجابية وسط مدينة السليمانية. لم يتم الإبلاغ عن أي وثائق نشر حتى الآن حول مرض التولاريميا في إقليم كوردستان. وثق هذا البحث أول كشف جزيئي لبكتريا F. tularensis في سلالات الأرانب المحلية في محافظة السليمانية إقليم كردستان العراق.

Introduction

Tularemia, also called as rabbit fever or deer fly fever, is a zoonotic disease caused by the facultative intracellular bacterium *Francisella tularensis* which is small, gram-negative, non-motile, aerobic cocobacillus. Tularemia was found in about 250 wildlife species, giving *Francisella tularensis* a wider host range than any other recognized zoonotic diseases (1). Various bloodsucking arthropods, for example ticks, tabanid flies, lice, mites, midges, fleas and mosquitoes, have been found naturally infected with the bacterium (2). One of the two species in the genus Francisella is called *F. tularensis* which is the only genus in Francisellaceae family, that belongs to Proteobacteria gamma-subclass (3) ,(4), (5). Type A strains are classified into two subpopulations: A1 (A.I. or A-east) and A2 (A.II. or A-west), each with four genotypes (A1a, A1b, A2a, and A2b) (6). Tularemia can be caused by both Type A and B strains in the United States, with Type A1b infections having a higher mortality rate than Type A1a, A2, and Type B strains (6), (7).

McCoy and Chapin discovered the causes of what they called a "plague-like disease of rodents" in 1911. Since their first samples came from Tulare County, California, they called the organism Bacterium tularense. Chapin was later stricken with a fever- illness, which forced him to skip work for twenty-eight days, after which his serum tested positive for antibodies against Bacterium tularense. Despite what appears to be an obvious correlation, no link between Chapin's disease and the rodent disease has been identified that time. Pearse also identified a human disease known as deer-fly fever in Utah at the same time. His theory was that the disease was transmitted by the deer-fly bite Chrysops discalis (8). Edward Francis isolated Bacterium tularense from deer-fly fever cases and local jack rabbits in Utah ten years later. He named the disease tularemia (9). Francis then demonstrated that Chrysops discalis could infect laboratory animals with Bacterium tularense (10). As a result, it was determined that the disease affecting California ground squirrels and humans in Utah had the same etiology and shared an arthropod agent capable of transmitting it. The bacterium would be assigned to the Pasteurella genus, and then transferred to Brucella on a temporary basis (11). It was proposed in 1947 that the bacteria be assigned to a new genus, Francisella, named after Dr. Edward Francis (12). The great and terrifying potential of *F. tularensis* as a biological weapon was not lost on twentieth-century world powers. The pathogen has many characteristics that make it an excellent candidate for offensive use. First it is one of the most infectious agents known to man, requiring only a small dose (less than 10 cells) to cause disease when inhaled (13). Second, since tularemia is not contagious among humans, an attack will be restricted to the initial target region. Third, given the pathogen's ability to survive in a variety of animal hosts, local reservoirs of the disease are likely to emerge, resulting in repeated outbreaks(14).

Although all strains of Francisella spp. are highly

infectious, there is great variety in the morbidity and mortality that each strain is able to induce in different host animals. *F. tularensis* is the most virulent bacterium in both humans and animals (15). Tularemia is difficult to diagnose clinically since the signs are nonspecific and often mimic influenza or other respiratory tract infections. It's also difficult to make a diagnosis in the lab. Culture procedures are difficult to conduct and are often avoided due to the fastidious nature of this slow-growing organism and, significantly, the high risk of inhalational contamination to laboratory staff (7). The most popular methods for diagnosis are serologic tests (including ELISA and agglutination assays), however antibody is not normally appeared until the fourteen days of the disease, and cross reactivity with other species is still a problem (16). *Francisella tularensis* has also been detected using immunohistochemical tests, fluorescent antigen scanning, and immune electron microscopy, but these methods are not widely available (13). In our research we used Polymerase Chain Reaction (PCR) because it is more sensitive and safe. Our aim in this research is to detect and diagnose tularemia in our country because it is one of the important zoonotic diseases.

Materials and methods

1. Area and animals of study:

The study carried out in four different places (Said sadq, Chwarta, Darbandikhan and Sulimani city), in Sulaimani province Kurdistan Region, Iraq. The study area was located 34-35ºN and 45-46ºE (figure 1). Sulaimani province has mild temperature and the previous rainfall season was 400-800 mm. The study was carried out between June and October 2020 and 100 local breed rabbits were tested. Age, sex and breed were detected.

Figure 1: This figure shows the areas of the study (green flags are the places were samples taken)

This study was conducted from June to October 2020. A total of hundred local breed rabbits (56 male and 44 female) were used in different geographical parts in Sulaimani governorate (table 2). Age (between 8 months to 2 years) and sex of animals were mixed and random. Blood (2ml) was taken from marginal vein of each animal using disposable needle (24 gauges) and

3ml syringes. Samples were kept in EDTA containing tubes and stored on ice upon delivery to the laboratory then stored at 4°C then were used to extract genomic material for further analysis.

DNA extraction and PCR

The AddBio® genomic DNA extraction kit for blood and tissue (South Korea) was used as written by manual of the manufacturer. Firstly, 25-35 mL blood was placed in a 1.5 ml plastic tube containing 200 µl lysis buffer (AddBio) then 20µl proteinase K (AddBio). Then they were incubated at 56 °C for 20 minutes in a dry bath, this was done for optimal tissue digestion. After that binding buffer (200 µl) was added (AddBio) and mixed by vortex. Further incubation at 56 °C for 10 minutes was needed. We then added and mixed 200 µl of molecular grade absolute ethanol (Merk). The mixture was then transferred into a spin column tube (AddBio) and centrifuged at 6000 x g for 1 min and the flow through were discarded and 500 µl wash buffer 1 (AddBio) was added, spun at 6000 x g for 1 min, flow through discarded, another 500 µl wash buffer 2 (AddBio) was added, centrifuged at 6000 x g for 2 min and the flow through was discarded. The column was then fixed in a new 1.5 ml Eppendorf tube and eluted with 200 µl ddH2O for 1 min incubation then 6000 x g spinning for 1 min. The spin column was discarded and the flow through is stored in -20 °C. Before storing, elutes DNA were measured by UV spectrophotometry absorbency at 260 and 280 nm (Eppendorf BioPhotometer).

The PCR was done beginning with (200 ng) extracted genomic DNA from samples. The PCR reaction was done in a 20 µl mix in a 200 µl PCR tube. The reaction mix was prepared in a clean hood (Pureair®). The reaction mix contained target DNA, 4 µl 10x PCR buffer (20 mM Tris-HCl, 50 mM KCl, pH8.4; AddBio), 2 µl of 50 mM MgCl2, 2 µl of 2.5 µM each dATP, dTTP, dCTP and dGTP (AddBio), 1.5 µl of 10 pM each primers (Genet Bio, South Korea), 2 units of DNA polymerase (platinum Taq DNA polymerase, AddBio) with specific primers for the bacteria as in (Table1) , primers taken after Versage et al 2003. The thermocycler used was a MyIQ (Biorad USA) machine.

To detect the *F. tularensis* DNA in blood of animals, a highly sensitive real time PCR technique had to be used. List of primers that were used in the PCR are shown in (Table 1). With specific primers for the two genes in (Table 1), a PCR reaction was performed using same parameters taken after (with appropriate primers and annealing temperatures (Table 1). Thermal conditions were as following: an initial denaturation at 94 °C for 10 min, then 40 cycles od of 94 °C for 30 sec, annealing step of 62 °C for 30 sec and an extension step at 72 °C for 45 sec, finished by a final extension at 72 °C for 3 min.
Results were analyzed using with software system of the IQ5 Monitor V2 (Bio-Rad laboratories) Figure 2, and the products were furthermore used in agarose gel electrophoresis to confirm band visualization (Figure 3).

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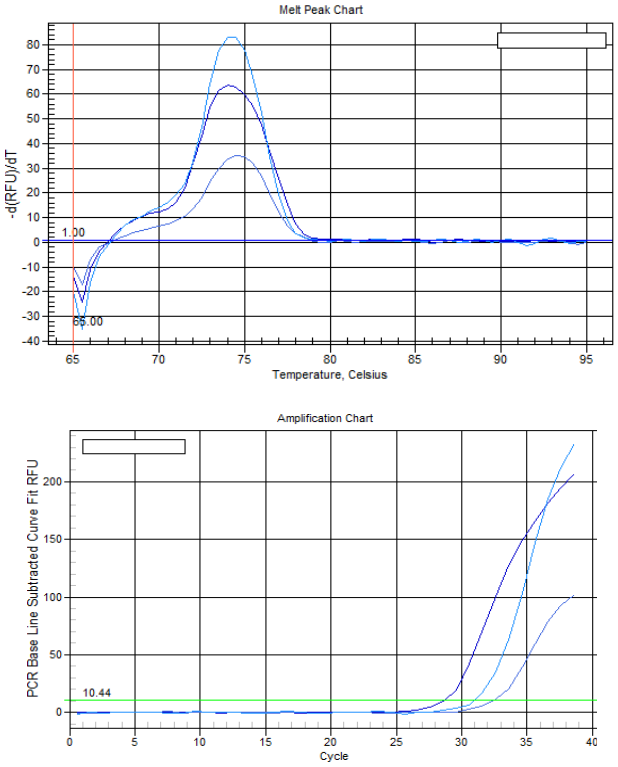

Figure 2. PCR Amp/Cycle Chart PCR Quantification (SYBR1).

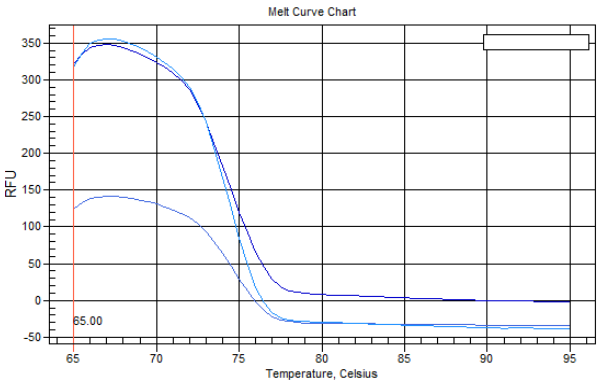

Figure 3. Melt Curve Chart and Melt Curve Peak Chart.

Table 1. Primers were used in conducting this study.

Primer Name	Sequence 5 -3	length	Tm	Amplicon size
ISFtu 2F	TTGGTAGATCAGTTGGTGGGATAAC	25 bp	62	97 bp
ISFtu 2R	TGAGTTTTACCTTCTGACAACAATATTTC	29 bp	61	

Results and discussion

To detect the *F. tularensis* DNA in blood samples, a highly sensitive real time PCR technique used. Only 16 out of 100 blood samples (16%) were positive taken from different local breed rabbits from four different places in Sulaimani province. All positive samples detected in the center of Sulaimani city. Out of 58 blood samples 16 samples were detected positive (27.59%). No positive samples were detected in Chwarta, Saidsadq and Darbandikhan (table 2 and figure 5).

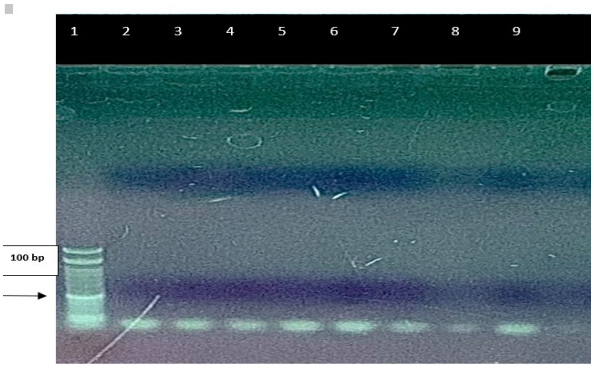

Figure 4. Agarose gel electrophoresis: lane 1: 100 bp DNA ladder as molecular size DNA marker, lane 2: positive control, lane 3-9 positive samples. Positive samples were run on 1% agarose gel, positive bands have 97 base pair size and located at 100 bp DNA ladder.

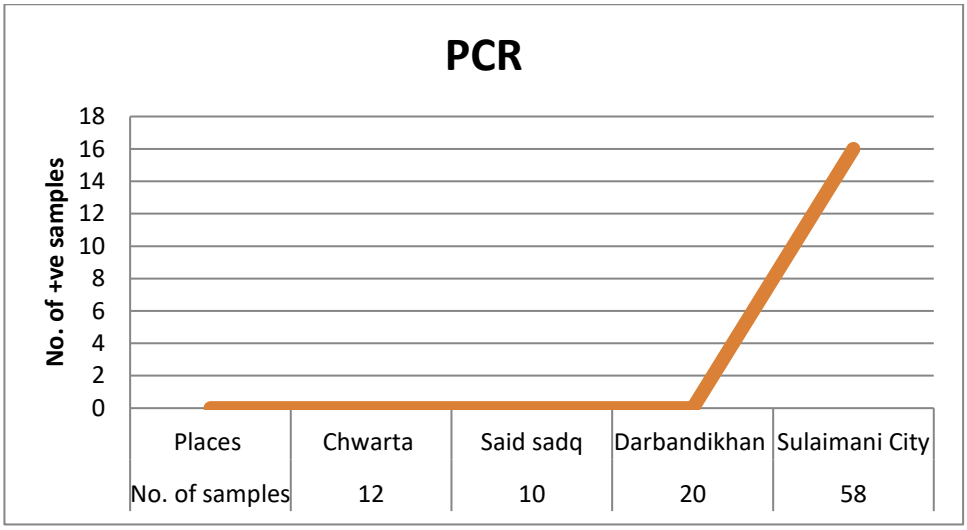

Figure 5: Shows number of samples taken from four different places with the positive and negative blood samples from a total of 100 domestic rabbits using PCR test.

Table 2: PCR results of examined blood samples in local breed rabbits according to places in Sulaimani province, Kurdistan-Iraq.

Number	Places	Blood samples	Age	Sex	Breed	PCR positive (%)
1	Chwarta	12	8 Months - 2 years	8 Males – 4 Females	Local breed	0 (%)
2	Said Sadq	10	2 Years	4 Males – 6 Females	Rex rabbit breed	0 (%)
3	Darbandikhan	20	1 Year	8 Males – 12 Females	Local breed	0 (%)
4	Sulaimani city	58	11 Months – 2 Years	36 Males – 22 Females	Local breed	16 (27.58%)
Total		100	8 Months to 2 Years	56 Males – 44 Females		16 out of 100 (16%)

DNA extraction and of *F. tularensis* detection in rabbits confirm that there are positive cases in Sulaimani province in the present study as no researches have been conducted in Iraq. Few studies were conducted on human tularemia in human beings in Iran and Turkey (14), (17). *F. tularensis* name was suggested by Edward Francis in 1919, which was a Bacterium tularensis, named after the country of Tulare, California where the diseases were endemic at that time among the rodents. It is a rare pathogen in most of the countries and it has been docuemted from several countries (18), (19).

This study was conducted in order to get a data about this disease which is capable to occur in humans and animals because it is a zoonotic disease (18). Blood samples were collected from local rabbits in Sulaymaniyah and its provinces to begin the study and explore it further more by using Real Time PCR to detect and isolate the positive samples from negative ones, this is when *F. tularensis* was documented in surrounding countries of Iraq such as Turkey and Iran, but there was not sufficient investigation and research about the disease in Iraq this is when the present study confirms positive samples were detected in Sulaimani province.

F. tularensis IgG antibodies in positive blood samples are a way to confirm the bacterium is present within the patient, the present study focused on samples that are taken directly from rabbits since there are not any other studies about the disease at all in both humans and animals. *F. tularensis* positive samples that are reported in the neighboring countries are also from blood serum (15), (9). In a study in Kurdistan Province, west-Iran, a seroprevalence research was conducted between 2011 and 2012 Analyzed 250 cases in Saryabad, Marivan, and Sanandai. Antibodies were discovered in 14.4% of the 250 cases (16), (15).

Comparing present study to the neighboring countries studies, illustrates the possibility of the same bacterium in Iraq as well. Having positive samples from rabbits' blood serum is telling that the disease might have been present in humans (20). However, since no other studies are conducted in humans cannot confirm the disease in humans in Iraq. In the meantime, sixteen samples (16%) out of 100 were detected as positive ones in the current study which explains there is a high probability of the disease in Iraqi Kurdistan individuals.

Conclusion

This current study confirmed the first detection of *F. tularensis* in local breed rabbits in Sulaimani Province, Kurdistan Region. More works need to be done for identification and detection of the disease in other domestic breeds as well as in humans. Possibility of transmission between human and animals could occur since it is documented a zoonotic disease and it is capable of causing issues to public health throughout the Kurdistan Region and Iraq.

Conflict of interest

Authors have no conflict of interest.

Acknowledgement

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