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Evaluate the Concordance Between Two Diagnostic Methods, RT-PCR And ELISA Techniques for the Detection of CCHFV in Karbala City

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Abstract:
Crimean-Congo haemorrhagic fever virus (CCHFV) is a widespread pathogen virus transmitted by ticks that causes a severe disease known as Crimean-Congo haemorrhagic fever in animals and humans. This study aims to investigate the occurrence of CCHFV in ticks and bovines in different regions of Karbala City/Iraq. Tick and blood samples were collected from four cattle farms, and molecular RT-PCR and serological analyses were conducted. The serum sodium concentration was calculated to compare infected and non-infected cattle with CCHFV. The study identified five tick genera, with Rhipicephalus (Boophilus) microplus and Amblyomma variegatum being the most prevalent species. However, no ticks tested positive for CCHFV. Serological analysis of the blood samples showed that none was positive for IgM antibody, but 19.00% tested positive for IgG antibodies and 29.3% for both IgG and IgM. The seroprevalence was higher in cattle from Ayn al-time (36.70%) and Hindiya (16.70 %) compared to other regions, the study found fair concordance between the two serological and molecular assays used for diagnosis of CCHFV virus; as well as the study concluded that sodium levels are an important prognostic factor for CCHFV infection, cattle with CCHFV.

Keywords: CCHFV, Ticks and ELISA, RT-PCR.

Introduction:

CCHFV is a virus in the Narrowvirus genus in the Bunyaviridae family. This agent causes Crimean-Congo hemorrhagic fever (CCHF), a severe and often fatal viral infection. The main route of infection is through the bite of infected ticks, especially the Hylomma and Rhipicephalus genus [1]. Crimean-Congo hemorrhagic fever (CCHF) is a viral infection caused by a nairovirus of the Bunyaviridae family. The disease is geographically widespread in Africa, the Balkans, the Middle East, and parts of Asia [2].

Tick bites mainly transmit this potentially fatal disease. CCHF outbreaks cause disease, and animal and human mortality ranges from 10% to 40%. Those who work with animals are most at risk, such

as slaughterhouses, farmers, workers, and veterinarians [3]. CCHF cases have been documented in West Africa, including fatal cases in Senegal and Mauritania. Various wildlife and domestic animals, including cattle, sheep, and goats, are hosts of CCHF without showing significant disease symptoms. Domestic cattle play an essential role in the virus's life cycle, acting as various sentinels to monitor CCHFV activity and identify potentially dangerous areas [4].

Seroepidemiological studies are needed to determine the prevalence of CCHFV infection and to define high-risk areas. Crimea-Congo hemorrhagic fever virus (CCHFV) has been isolated from a variety of sources in sub-Saharan West Africa, including ticks, wild and domestic vertebrates, and humans in areas such as Senegal, as well as in community-based studies including southern Iraq, e.g. [5] reported that among 112 cases of human Crimea-Congo hemorrhagic fever virus (CCHFV), 97 cases (46%) were confirmed by reverse transcriptase polymerase chain reaction (RT-PCR). In comparison, 115 cases (54%) were suspected cases and geographically reported the majority of confirmed cases, accounting for about 48% of the total in Dhi Kar district, and [6] reported cases of Crimean_Congo Hemorrhagic Fever Virus (CCHFV) in March 2022 It occurred, and in June 2022, cases are still being reported and it was determined that the majority of cases, 142 individuals (64.8%; in general), were from southern Iraq.

Several studies have reported that hyponatremia (low serum sodium levels) is commonly observed in patients and animals with CCHFV infection; in a prospective study of CCHFV patients, [7] found that hyponatremia was present in 86.8% of the patients. Patients with severe hyponatremia (sodium serum levels <125 mmol/L) had a significantly higher mortality rate compared to those with mild or no hyponatremia. Few studies have been conducted on the spread of the Crimean-Congo hemorrhagic fever virus (CCHFV) in Iraq, especially in Karbala. Understanding the geographic distribution of ticks and CCHFV epidemiology in livestock is essential due to its important role in the infection cycle [8]. This highlights the need for comprehensive surveys to characterize the spread of CCHFV, especially in the main breeding areas of Karbala, where ticks and cattle play an essential role in virus transmission. Comparatively, evaluate enzyme-linked immunosorbent assays (ELISAs) and serum sodium levels as predictors of CCHFV diagnosis in animals.

Materials and methods:

Study regions:

This cross-sectional study occurred in four separate locations in the city of Karbala, representing different areas. Four farms were selected as sampling sites for tick collection and bovine blood, representing the AL Hur, Hindia, Al-Husseinia, and Ayn Al-Tamar areas. These samples were sent to the veterinary laboratory medicine at the University of Karbala between March and November 2022 to diagnose “Crimean_Congo haemorrhagic fever virus (CCHFV)” as part of the ongoing surveillance efforts for haemorrhagic fever viruses in Karbala City, Iraq (Figure 1).

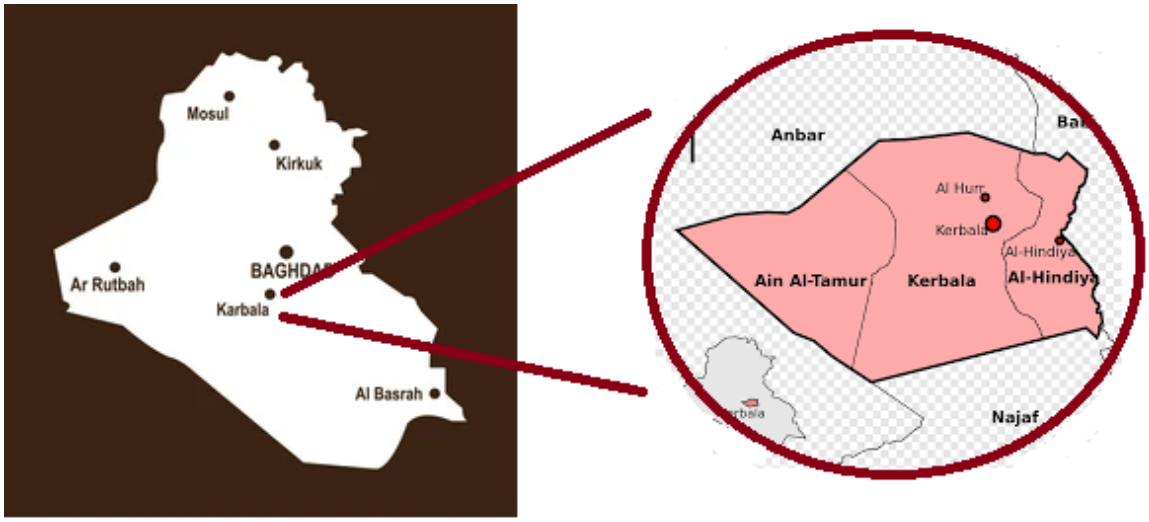

Figure 1: study region in Karbala city in Iraq country.

Sample collection:

A total of 480 ticks and 120 cattle blood samples were obtained from four cattle farms located in Karbala city. The towns consist of four regions: Al-Hurr, Al-Hindiya, Al-Husseiniya, and Ain-Altamor. Thirty bovines from each farm were selected for the study, and tick and blood samples were collected. Ticks were collected from randomly chosen animals of various age groups, with four ticks collected from each bovine. The tick collection process involved a thorough search across the entire body, focusing on areas preferred by ticks, such as udders, testicles, ears, and near mucous membranes in the anogenital distance. After collection, the ticks were individually placed in labelled cap tubes for proper identification. Blood samples were collected in gel tubes by puncturing the jugular vein. Following centrifugation, the serum was refrigerated at four °C and subsequently sent to the medicine laboratory at the University of Karbala for further analysis.

Ticks were identified using binocular magnifiers microscopic set at 10X and 20X magnification, along with dichotomous identification keys [9]. Once identified, the tick batches from each farm were stored in a liquid nitrogen canister before transportation. For long-term storage, the ticks were kept in a freezer at -80°C.

Morphological classification:

One type of tick was collected from each animal and taken to the Natural History Museum and Research Center in Baghdad, Department of Insects and Invertebrates, for morphological classification of ticks.

Molecular detect of CCHFV in ticks

RNA was extracted from the ticks, about 500 µl of phosphate-buffered saline buffers were added to the ticks in new 1.5 microcentrifuge tubes, and the mixture was crushed using an automatic grinder called FastPrep-24TM5G/ china. After centrifugation, the resulting supernatants were carefully collected in 1.5 ml microcenterfuge tubes. For RNA extraction, 140 µl of the supernatant obtained from the tick samples and 140 µl of bovine serum were collected. The AddPrep Viral Nucleic Acid Extraction Kit from Korea was employed for the RNA extraction process following the instructions

provided by the manufacturer. The extracted RNAs were eluted and stored at -20°C for further analysis.

For the molecular identification of the CCHFV virus, Real_time RT_PCR (One-step Promega/USA) was conducted using AddScript RT-PCR SYBR Kit, The AddScript RT_PCR SYBR Kit method involves preparing a PCR tube with components such as nuclease-free water, buffer, primers, optional ROX dye, RNA template, and AddScript Enzyme Solution. The reaction is subjected to cDNA synthesis, initial denaturation, PCR cycling, and melting analysis, the amplified of the S segments of the CCHFV were performed using specific primers and a probe. The sense primers (CCHF S1) were designated as 5-TCT CAA AGA AAC ACG TGC C-3 , the antisense primers (CCHF S122) were designated as 5- CCT TTT TGA ACT CTT CAA ACC-3 , and the probe (CCHF probe) was designated as FAM-ACT CAA GGKAAC ACT GTG GGC GTA AG-BHQ1 [10].

The RT-PCR reaction was carried out in a 25 µl volume, which included 1 µl of the extracted RNA, 2x Quantitect Probe, nuclease-free water, and the primers and probe. The amplification program consisted of a reverse transcription phase at 50 °C for 10 minutes, followed by a denaturation phase at 95 °C for 15 minutes. This was followed by 40 cycles of amplification at 95 °C for 20 seconds and 60 °C for 1.5 minutes.

Immunological assay for detection CCHFV

The serological analysis for detecting antibodies to CCHFV involved using ELISA (biotek / USA). For IgM detection, bovine IgM antibodies were immobilized on a microplate using bovine Crimean-Congo hemorrhagic fever virus IgM (Abbexa LTD, Cambridge, UK) and incubated with serum samples. Specific viral and control antigens were added, and the occurrence of antibodies to the virus caused immune complex formation. Bovine "Crimean_Congo hemorrhagic fever virus IgG" (Abbexa LTD, Cambridge, UK) detection used the same plate with viral antigen immobilization, and serum samples were added. Peroxidase-labeled bovine IgM conjugate was used for both IgM and IgG detection. Plates were read using spectrophotometry, and the qualitative determination of CCHF-IgG was determined by comparing it with the CUTOFF value.

Measurement of total sodium serum levels

Plasma was separated from Li-heparinized whole blood by centrifugal at 3000 rpm for 10 minutes at 20°C using a Megafuge 3.0R. Hemolyzed plasma samples were excluded. Sodium concentrations were measured in the plasma samples using Flame atomic absorption/emission spectroscopy (SOLAAR M6, Thermo Fisher) operated in emission mode.

Statistical analysis:

Descriptive statistics were conducted using SPSS version 25.0 to analyze the characteristics of the study population and laboratory results. The qualitative variable was presented as numbers and percentages. Roc curve analysis determined the cut-off sodium levels between serum-infected and non-infected cattle with CCHFV.

Results and Discussion:

Table 1 shows the occurrence of Crimean Congo Hemorrhagic Fever Virus (CCHFV) infection across different regions of Karbala city with relatively low prevalence:

Table 1: occurrence of CCHFV in ticks isolated in Karbala regions

Region	Sample Size	Positive Cases	Prevalence	95% CI
AL Hur	30	1	3.30%	0.1% - 17.2%
Hindiya	30	5	16.70%	5.6% - 34.7%
Al-Husseiniya	30	0	0.00%	0.0% - 11.6%
Ayn al-tamr	30	11	36.70%	19.9% - 56.1%
Total	120	17	14.20%	8.5%-21.7%

The RT-PCR analysis detected CCHFV in 17 of the 120 tick samples tested (14.2% positivity rate); all positive samples had Ct values ranging from (32-34) Figure 2, indicating the presence of CCHFV genetic material in these ticks. On the other hand, the remaining 103 tick samples (85.8%) were negative for CCHFV and, therefore, do not have associated Ct values to report.

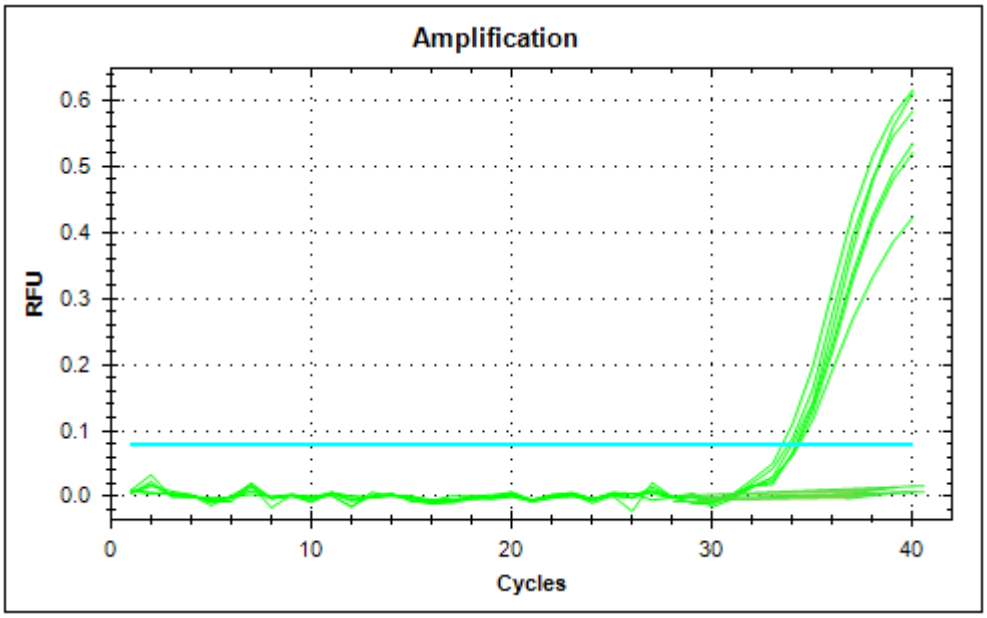

Figure (2) amplification plot of CCHFV in some positive and negative ticks isolated from cows in Kerbala City

The study found that most cases of the hemorrhagic fever virus were detected in ticks belonging to the Rhipicephalus (Boophilus) microplus and Rhipicephalus (Boophilus) decoloratus species. The prevalence of the virus was 35.3% in the ticks of the Rhipicephalus (Boophilus) microplus species and 29.4% in the ticks of the Rhipicephalus (Boophilus) decoloratus species Table 2.

Table 2 showed number and percentage of five classes of ticks carry CCHFV by using molecular assay

Tick Class	All Ticks +	Prevalence	%
Rhipicephalus (Boophilus) annulatus	17	3	17.60%
Rhipicephalus (Boophilus) decoloratus	17	5	29.40%
Rhipicephalus (Boophilus) geigyi	17	2	11.80%
Rhipicephalus (Boophilus) microplus	17	6	35.30%
Nymphe	17	1	5.90%

On the other hand, the study recorded the prevalence of immunological ELISA kit for quality detection of CCHFV in cows in Karbala city; sero epidemiological research was conducted, where 120 bovine serum samples were tested for IgM and IgG antibodies against Crimean-Congo Hemorrhagic Fever Virus (CCHFV) table 3.

Table (3) Qualitative ELISA IgM and IgG for detecting cows infected with CCHFV in Karbala city.

Antibody Profile against CCHFV	Number of Cows	Percentage
IgM positive only	0	0.00%
IgG positive only	11	19.00%
IgG and IgM positive	17	29.30%

The kappa statistic measures the agreement between the two tests: 17 cows were detected as CCHFV-positive by RT-PCR, 28 cows were detected as having current/recent CCHFV infection by ELISA, and the concordance between the two tests, as measured by the kappa statistic, is 0.3636, indicating a fair to moderate level of agreement table (4).

Table (4) The kappa statistic measures the agreement between two tests

	RT-PCR Positive	RT-PCR Negative	Total
ELISA Positive	17	11	28
ELISA Negative	0	92	92
Total	17	103	120

ROC curve analysis for prognostic sodium levels:

ROC curve analysis for prognostic sodium levels in cattle infected with CCHFV was measured to determine the predictor variable of sodium levels, and the outcome variable would be the survival status (infected cattle that survived and non-infected with CCHFV); the ROC curve will depict a curve that is closer to the top-left corner, indicating higher sensitivity and specificity. The area under the curve (AUC) representing the overall prognostic test was recorded as 0.85. A higher AUC value indicates a better predictive ability for measurement of bovine cattle infected with CCHFV figure (3) and the actual value obtained for each measure (AUC: 0.85, Cutoff Point: 134.5) and the range of value within with 95% confidence (AUC: 0.78 - 0.92) Table (5).

Table (5): Measurement of sodium serum concentration in serum of cattle

Minerals	Measure	Value	95% Confidence interval	
Sodium levels mmol/L	AUC	0.85	0.78	0.92
	Cutoff Point Cattle infected with CCHFV	<134.5	129.6	163.82
	Cutoff point Cattle no infected with CCHFV	≥ 134.5		
Sensitivity	88%		81%	92%
Specificity	86%		79%	90%

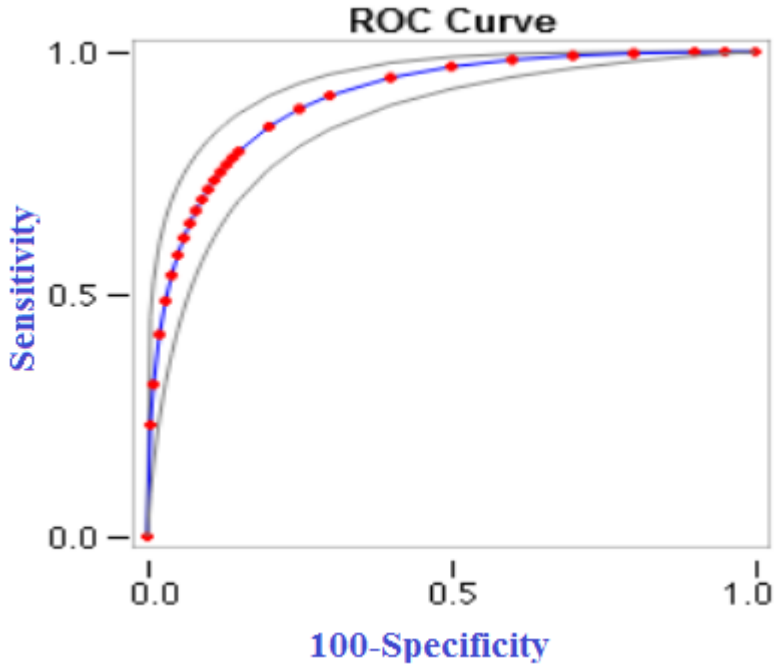

Figure (3): receiver operating characteristic (ROC) curves for determination of sodium levels in serum cattle

Discussion:

CCHFV is endemic in parts of Asia, Africa, Eastern Europe, and the Middle East; the virus is found in regions where tick vectors and susceptible animal hosts are present [11]. The results of the current study showed that there were about 14.2% (95% CI; 8.5%-21.7%) of hemorrhagic fever infections in the city of Karbala, Iraq, and the highest infection rate was in the Ain al-Tamar region, which included about 36.7 % with (95% CI; 19.9% - 56.1%) of the total infections compared to the Karbala city. The study disagreed with [5], who showed that 97 cases (46%) were confirmed positive for CCHFV through "reverse transcriptase polymerase chain reaction (RT-PCR)" testing, and 115 cases (54%) were classified as suspected CCHFV cases but did not have RT-PCR confirmation.

The Natural History Museum in Baghdad and the Department of Invertebrates classified the ticks, and five species belonging to the ticks found in Iraq were obtained. The study noticed that most cases of the hemorrhagic fever virus were found in ticks of the Rhipicephalus (Boophilus) microplus and Rhipicephalus (Boophilus) decoloratus species, and the percentage in their entrails was 35.3 % and 29.4%, respectively. The study disagreed with [12], who recorded that all ticks in his study do not carry viruses in their bodies. No ticks were positive among 1,174 ticks collected from spur_thighed tortoises in Tunisia.

The current study examined antibody prevalence for Crimean-Congo Hemorrhagic Fever Virus (CCHFV) in Karbala region cattle. The results showed that out of the total cattle sampled (120) cases, The study found that 11 animals developed only CCHFV-specific IgG antibodies, indicating prior infection with the virus. No animals showed CCHFV-specific IgM antibodies alone, indicating recent or acute illness. In addition, 17 animals were found to have both CCHFV-specific IgG and IgM antibodies; no presence of IgM antibodies suggests recent or active CCHFV infections in our study, while the IgG antibodies indicate past exposure and potential immunity [13]. The detection of both IgG and IgM antibodies to CCHFV in the same sample may indicate reinfection or reactivation of previous CCHFV infection rather than a primary malignancy [14].

The overall CCHFV seroprevalence of 29.30% observed in the Karbala cattle population is higher than the rates reported in Niger (9%) [14] and Egypt (3.83%) [16], and Mali (66%) [17] and the highest seroprevalence in animals from Mauritania (67%) [18]. The kappa statistic indicates the concordance between two diagnostic tests, in this case, ELISA and RT_PCR, for detecting "Crimean-Congo hemorrhagic fever virus (CCHFV)." A kappa value of 0.36 indicates a "fair" level between the two tests; this shows that there are notable differences in the results of ELISA and RT_PCR for the detection of CCHFV and that ELISA and RT_PCR do not correlate well in detecting CCHFV positive samples. False positives or negatives can result when comparing two methods [19]. The accuracy and reliability of using ELISA or RT-PCR alone to diagnose CCHFV may be limited, as they do not always give the same results, and RT-PCR detects the presence of viral genes there, which generally increases during the acute phase of infection. While seropositive ELISAs detect antibody production, which takes time to develop after infection, may not occur initially during the phase, on the other hand, genomic variation in CCHFV strains can affect the performance of RT-PCR and ELISA, as virus-specific assay systems or epitopes can be developed [20]. The test objectives may also be inconsistent with circulating CCHFVs, leading to inconsistent results between the two tests.

Sodium levels are an essential predictor of CCHFV infection; animals with CCHFV infection with low sodium levels had poorer prognosis and clinical outcomes than those with high sodium levels. It is low. This study is consistent with [21], who determined that electrolyte imbalances, including hyponatremia, are common in patients infected with CCHFV and found that low serum sodium levels were associated with disease severity increase associated with increased mortality.

The association between hyponatremia (low sodium levels) and poor prognosis in CCHFV infection highlights the importance of closely following electrolyte imbalance as clinical management of this viral hemorrhagic fever in animals [7]. The predictive value of sodium levels can help veterinarians identify high-risk animals with CCHFV as well. Improvements in nomenclature may inform the development of appropriate treatment strategies.

In conclusion, the study concluded that there was fair concordance between the two serological and molecular assays used to diagnose CCHFV infection.

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