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Molecular detection of coronavirus in camelids and bovines using real-time quantitative polymerase chain reaction in Wasit Province, Iraq

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ABSTRACT

Background: Coronaviruses (CoVs) are a diverse group of RNA viruses that cause respiratory and gastrointestinal diseases in humans and animals. Over the past two decades, outbreaks of Middle East Respiratory Syndrome Coronavirus (MERS-CoV) and bovine coronavirus (BCoV) have affected animal populations, especially in regions with close animal-human interactions, such as the Arabian Peninsula and Iraq. Given the potential for zoonotic transmission, understanding the prevalence and spread of CoVs among livestock is essential for managing potential risks to animal and human health.

Aim: This study aimed to investigate the prevalence of MERS-CoV in camels and BCoV in bovines within the Wasit Governorate of Iraq to assess the infection rates and potential interspecies transmission risks.

Methods: One hundred and fifty nasal swab samples (75 from camels and 75 from bovines) were collected between November 2022 and April 2023. The samples were analyzed for the presence of MERS-CoV and BCoV using real-time quantitative reverse transcription PCR (qRT-PCR) targeting the nucleocapsid (N) gene for each virus. Standard procedures for RNA extraction were followed, and qRT–PCR assays were conducted using specific primers to ensure high sensitivity and specificity.

Results: MERS-CoV was present in (42%) of the camel samples, whereas BCoV was detected in (34%) of the bovine samples. Statistical analysis indicated a significant difference ($p < 0.05$) in infection rates between camels and bovines, with a higher prevalence observed in camels. The clinical signs observed in infected camels included fever, nasal discharge, and appetite loss, whereas infected bovines exhibited symptoms such as diarrhea and respiratory distress.

Conclusion: The high prevalence of MERS-CoV and BCoV in camels and bovines in the Wasit region indicates a substantial risk for the continued spread of these viruses within animal populations. These findings underscore the importance of surveillance and biosecurity measures to control the spread of coronavirus among livestock, potentially reducing zoonotic transmission risks. Further research is required to understand the transmission dynamics of CoVs in mixed livestock farming systems.

Keywords: Coronaviruses, MERS-CoV, BCoV, RNA viruses, qRT-PCR, zoonotic transmission.

Introduction

Coronaviruses (CoVs) cause disease in humans and animals (Saied *et al*., 2021). In the past 20 years, three global outbreaks have occurred: Coronavirus Disease-2019 in Wuhan, China; Middle East Respiratory Syndrome Coronavirus (MERS-CoV) in Saudi Arabia; and Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) in China (Colina *et al*., 2021). Dromedaries are widely recognized as natural hosts of MERS-CoV, which can infect humans and domestic animals (Kandeil *et al*., 2019). While dromedary camels represent the virus reservoir and a means of human transmission, bats are believed to have been the virus's original host (Al-Shomrani *et al*., 2020). MERS-CoV infection in humans may persist due to intimate interactions between infected dromedaries and humans, facilitating zoonotic transmission (De Wit *et al*., 2016). Bovine coronavirus (BCoV) causes neonatal calf diarrhea in newborn calves, winter dysentery in adult bovines, and respiratory tract infections in all ages (Johnson and Pendell, 2017). Bovine respiratory coronavirus (BRCoV) is a strain of coronavirus isolated from the respiratory tract, whereas bovine enteric coronavirus (BECoV) is a strain of coronavirus isolated from the intestinal tract. The BECoV strains are responsible for calf diarrhea, and winter dysentery are further classified as BECoV-CD and BECoV-WD, respectively (Boileau and Kapil, 2010).

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CoVs belong to the Nidovirales order and the subfamily Coronavirinae of the Coronaviridae family (Lai *et al*., 1994). The Coronavirinae subfamily is divided into four genera: Alphacoronavirus, Betacoronavirus, Gammacoronavirus, and Deltacoronavirus (α-, β-, γ-, and δ-CoV) based on genomic structure and evolutionary relationships. CoVs from mammals are generally included in α-CoVs and β-CoVs, while CoVs from birds are mainly included in γ-CoVs and δ-CoVs-CoVs (Li *et al*., 2022). The α-CoVs and β-CoVs are of greater interest due to their ability to penetrate animal-human barriers and cause significant human infections (Coleman and Frieman, 2014). CoV chimeric are enclosed viruses with the most giant known RNA virus genomes, with a single-stranded, positive-sense RNA genome ranging from 26 to 32 kilobases (Weiss and Navas-Martin, 2005). Electron microscopy revealed spike projections from CoV virions resembling a crown, or corona in Latin, which is the term used to describe the virus (Pal *et al*., 2020). All CoVs have identical genomic structures and expressions, with structural proteins, such as spikes, envelopes, membranes, and nucleocapsid, originating from the open reading frame 1a/b-encoded nsp1 through nsp16 (Su *et al*., 2016). The hemagglutinin-esterase accessory protein, which is found exclusively in certain beta-CoVs, forms a smaller layer of projections within the betacoronavirus genome (Masters, 2006). The viral envelope's helical capsid structure, formed by nucleocapsid protein binding with genomic RNA, creates spikes in some CoV virions, while transmembrane proteins "M" and "E" aid virus assembly (Weiss and Leibowitz, 2011).

Real-time quantitative polymerase chain reaction (RT-qPCR) is frequently utilized for detecting gene expression levels and diagnosing acute respiratory viral infections quickly (Corman *et al*., 2020). Conventional real-time RT-PCR assays require large laboratory instruments and take approximately 2 hours to amplification (Shirato *et al*., 2020). MERS-COVs and BCoVs can be diagnosed in the laboratory by detecting their genes in clinical samples from diseased animals (Decaro *et al*., 2008; Shirato *et al*., 2020). The standard method for diagnosing MERS-CoV is RT-PCR (Al Johani and Hajeer, 2016). Cattle rectal and respiratory samples can be used to detect and identify BCoV and BCoV-like RNA using RT-PCR–based techniques (Erles *et al*., 2003; Takeuchi *et al*., 2006).

The Iraqi government and official institutions have shown limited or no interest in the surveillance or vaccination efforts for MERS-CoV and BCoV. Currently, there are no established national programs targeting these viruses. Efforts in this field are mainly driven by individual researchers, focusing on specific aspects, such as the partial genetic or immunological characterization of CoVs in camels and cattle. This highlights the need for greater institutional involvement to address potential public health and veterinary concerns. In general, viral diseases in veterinary medicine are a significant threat to animal health and can cause considerable economic losses. Recent studies conducted in southern Iraq highlight the importance of understanding these diseases from various perspectives (Thwiny *et al*., 2015; Thwiny, 2016; Thwiny *et al*., 2018; Al-Mubarak *et al*., 2022; Al-Mubarak *et al*., 2023). Together, these studies emphasize the need for comprehensive approaches to manage viral diseases in veterinary medicine in southern Iraq.

This study aimed to investigate the presence of CoVs in camel and bovine samples through real-time quantitative polymerase chain reaction (qPCR) analysis, with the objective of elucidating viral prevalence and identifying potential risk factors associated with transmission within these animal populations.

Ethical approval and animal welfare statement

This study was conducted according to ethical guidelines and was approved by the Scientific Committee of the College of Veterinary Medicine, University of Basrah (Approval Number:39/2024). All procedures involving animals complied with institutional and international standards for animal welfare. Efforts were made to minimize animal suffering and ensure proper care during the study.

Materials and Methods

Sample collection

The methods of sample collection in this study were as follows: Nasal swabs were collected from 75 camels to detect viral shedding from the respiratory tract. In addition, nasal and rectal swabs were collected from 75 bovines to detect respiratory and gastrointestinal shedding of the virus. We obtained samples from animals across different age groups in Wasit Province, Iraq, between November 2022 and April 2023. Sample collection followed standard protocols to minimize animal stress and discomfort, ensuring ethical considerations were adhered to during the process. We collected swabs from all hosts using a virus sampling kit (Jiangsu, China). We stored the samples on ice during transportation to the microbiology laboratory in the College of Medicine, University of Wasit, where they were stored at −80°C until they were thawed and used for RNA extraction and CoV testing.

We have automated RNA extraction

Coronavirus RNA was extracted automatically using a nucleic acid extraction kit (magnetic bead method) from Chongqing, China. We added 15 µl of proteinase K to mix wells A1–H1 and A7–H7, followed by 200 µl of viral transport medium sample, following the manufacturer's guidelines. The plates were placed in the EXM3000 isolation system, and the extraction process was allowed to run for 9 minutes.

qRT-PCR

Following the manufacturer's instructions, a GoTaq®1-Step RT-qPCR System Promega kit (Wisconsin, USA) was used for a one-step qRT–PCR assay. The final GoTaq® 1-Step RT-qPCR reaction mixture for qRT-

PCR consisted of 10 µl of GoTaq® qPCR Master Mix 2×, 2 µl of forward primer 10×, 2 µl of reverse primer 10×, 0.4 µl of GoScript™ RT Mix for 1-Step RT-qPCR, 4 µl of extracted RNA, and up to 20 µl of nuclease-free water. The mixture underwent one reverse transcription phase for 15 minutes at 38°C, an initial denaturation step of 10 minutes at 95°C, and 40 cycles of 10 seconds at 95°C, 30 seconds at 58°C, and 30 seconds at 72°C.

Primers

For qRT-PCR, we used oligonucleotide primers targeting two distinct genomic sites: the *gene* fragment specific to MERS-CoVs in camels. Based on the nucleocapsid gene's start codon, the primer sequence was as follows: the upstream primers 5'-TGCAAGCTTTTGGTCTTCGC-3' and 5'-AGCAAGCTCAGCAATTTGGG-3' are used to synthesize the desired product (Al Salihi, 2017). The *N gene* fragment for the Mebus strain in bovines was sequenced using the upstream primer 5'-GCAATCCAGTAGTAGAGCGT-3' and the downstream primer 5'-CTTAGTGGCATCCTTGCCAA-3' are both essential components in the qRT-PCR synthesis process (Hasoksuz *et al*., 2002).

Statistical analyses

The data were analyzed using SPSS software and Chi-square tests to establish the relationship between MERS-CoV in camels and BCoV in bovines and calculate the percentages of infected animals.

Results

Clinical cases were previously diagnosed using clinical findings from diseased animals such as camels and bovines (Table 3 and Fig. 1). Clinical findings revealed fever, ocular, and nasal discharge, and appetite loss in camels aged 4–14 years (Table 1 and Fig. 3). During data collection and case history gathering from camel owners, it was clarified that there was an unlimited number of fetuses with gray eyes at different stages of gestation in pregnant females. Additionally, it was noted that the camel mortality rate during the study period was (3%) during the study period. The disease in bovines manifests as soft, runny, and weak diarrhea in calves aged 7–21 days, green watery diarrhea in calves aged 3–9 months, and persistent cough and nasal discharge in cows aged 4 years (Table 2 and Fig. 2).

Discussion

This unique study in Iraq aimed to explore camels and bovines as hosts of the coronavirus, which causes various diseases, such as respiratory and digestive diseases in camels. The new study's results and previous research are entirely out of the ordinary; the study revealed a substantial increase in the coronavirus infection rate in camels and bovines (Alrodhan, 2017; Mansour *et al*., 2013). This phenomenon dates back to the period accompanying our study of the advanced strategic shifts that characterize the coronavirus, which usually increases its spread and the strength of its infection. However, some coronavirus genera pursue a niche evolutionary strategy to infect a specific host and bypass others, as occurs in gammacoronavirus, which only infects birds.

According to recent molecular and serological evidence, the dromedary camel is the host of MERS-CoV. Before 2012, MERS-CoV or MERS-like CoV was circulating among dromedaries in Africa and the Arabian Peninsula (Reusken *et al*., 2014). RT-PCR confirmed the presence of MERS-CoV in nasal samples, live and lung tissue samples, and carcasses from dromedary camels in various Arabian Peninsula locations as evidence (Khalafalla *et al*., 2015). As previously mentioned, MERS-CoV was confirmed in camel samples by screening them with MERS-CoV-specific RT-PCR targeting the N gene (Corman *et al*., 2012). Research on camels has shown that MERS-CoVs are prevalent in various countries (Alagaili *et al*., 2014; Chu *et al*., 2014; Hemida *et al*., 2014; Wernery *et al*., 2015). The first Iraqi study using RT-PCR to detect and characterize MERS-CoV in dromedaries and humans found MERS-CoV present in (15%) and (5%) of the population, respectively (Al Salihi, 2017).

Previous research has focused on genetic characterization and the prevalence of MERS-CoVs in Qatar (1.9%), UAE (1.6%), Egypt (15.4%), Jordan (93.3%), Kenya (0.9%), and Pakistan (2.8%) (Raj *et al*., 2014; Yusof *et al*., 2015; Ali *et al*., 2017; van Doremalen *et al*., 2017; Ommeh *et al*., 2018; Zohaib *et al*., 2018). This study found that (42.6%) of camel nasal swabs were detected by qRT-PCR, contradicting previous findings suggesting different MERS-CoV infection rates between 2014 and 2018, with the highest rate in Jordan in 2017. The paucity of studies and the absence of prospective surveillance of MERS-CoV in camels in Iraq contribute to our imperfect

Table 1. Sample collection overview: Nasal swabs from camels by age group.

Sample ID	Age group	Type of sample	Host	No. of sample	No. of positive sample
1	4–7 years	Nasal Swab	Camel	14	2
2	7–9 years	Nasal Swab	Camel	37	10
3	Older than 9 years	Nasal Swab	Camel	51	20
Total				75	32

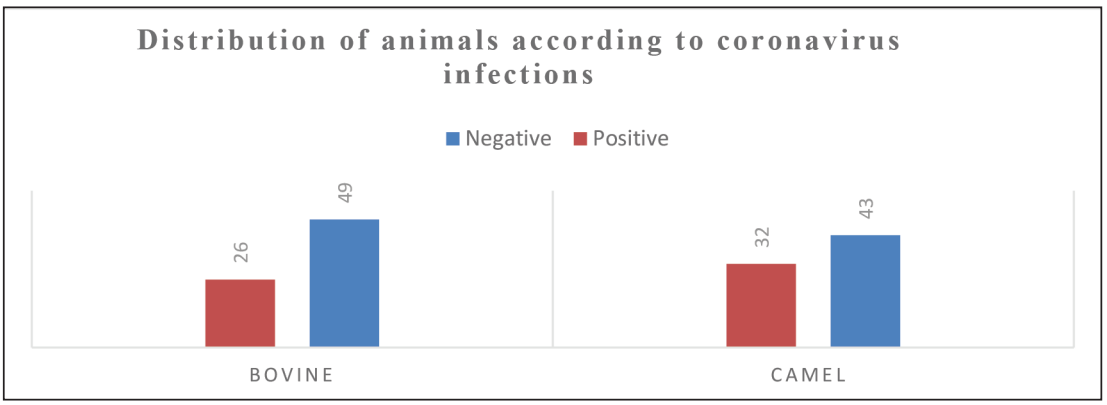

Figure 1. The study examines the distribution of animals based on infection by BCoVs and MERS-CoVs.

Table 2. Sample collection overview: Rectal and nasal swabs from cattle by age group.

Sample ID	Age group	Type of sample	Host	No. of sample	No. of positive sample
1	3–21 days	Rectal Swab	Calf	43	17
2	3–9 months	Rectal Swab	Calf	27	9
3	4–7 years	Nasal Swab	Cow	5	0
Total				75	26

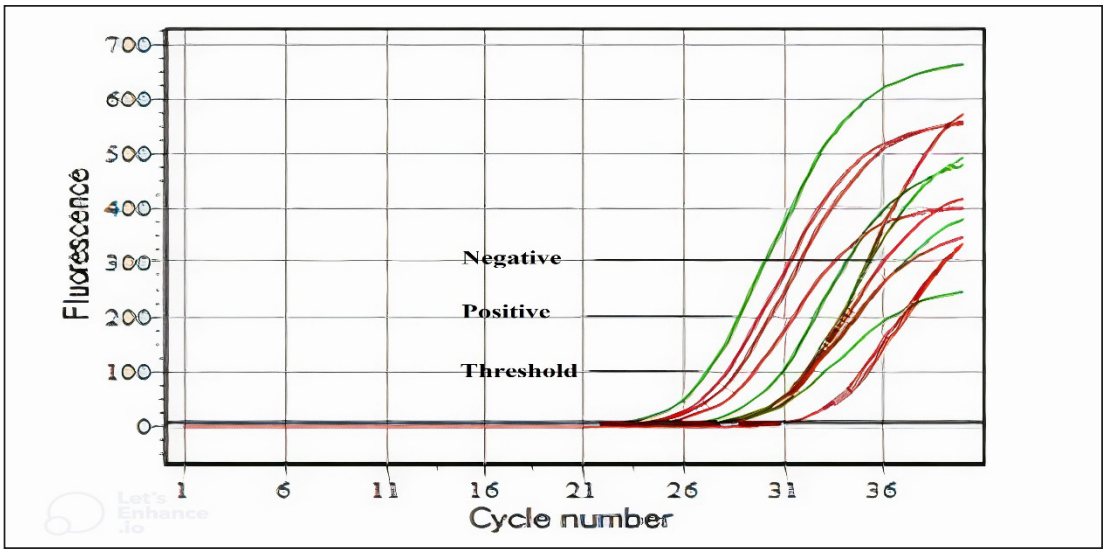

Figure 2. Amplification plot curves in bovine indicate a correlation between the fluorescence of the Fluorescein Amidite (FAM) channel and cycle number. The threshold, positive, and negative samples were tested using the RT-qPCR device. The threshold based on a positive sample ranged from 28 to 31 cycles.

understanding of the virus's epidemiology and the risk factors associated with its spread. However, when comparing the results of this study in Iraq with earlier data (Al Salihi, 2017), it is evident that the prevalence of MERS-CoV has increased over time.

Owners of camels in Badra, Iraq, reported no disease, but during grazing in the Middle Euphrates, they experienced high fever, abortion, and stillbirth. A previous study revealed that camelid infections have a high transmission potential and are characterized by widespread viral shedding and upper respiratory tract replication (Te *et al*., 2022). Based on the findings that MERS-CoV is typically shed from diseased camels through nasal discharge and remains viable and environmentally stable on various surfaces under different temperatures and humidity levels, particularly

Table 3. Animal distribution based on coronavirus infection rates.

Infection status	Camels		Bovines		*p* Value
	Number	Percentage %	Number	Percentage %	
Negative	43	57.3%	49	65.3%	0.001
Positive	32	42.6%	26	34.6%	
Total	75	100.0%	75	100.0%	

*The association was significant at the 0.05 level.

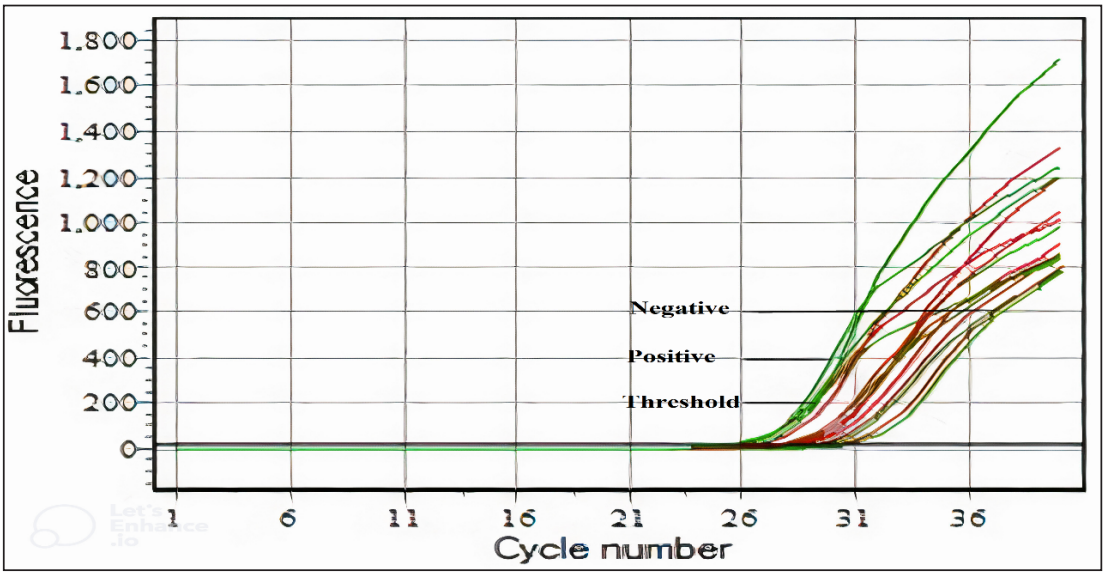

Figure 3. The amplification plot curves in camles indicate a correlation between the fluorescence of the FAM channel and the cycle number. The threshold, positive, and negative samples were tested using an RT-qPCR device. The threshold based on a positive sample ranged from 27 to 31 cycles.

at lower temperatures and humidity (Van Doremalen, 2013), and considering the practices of camel owners who rotate their herds in specific grazing areas. We hypothesize that virus shedding from camels, such as through nasal discharge, contributes to the environmental persistence of MERS-CoV on grass or feed. The virus remains viable, stable, and infectious on contaminated grass, facilitating its transmission. This transmission occurs through the ingesting or inhalation of contaminated grass between camel herds, demonstrating the virus's environmental stability and its role in facilitating interherd transmission.

MERS-CoVs spread among dromedaries, and their strains exhibited high genetic similarity to human isolates (Omrani *et al*., 2015). Moreover, sheep, goats, and donkeys grown close to camels tested positive for MERS-CoV (Kandeil *et al*., 2019). Recent studies have shown that dromedaries play crucial roles in the spread of MERS-CoV at the animal-human interface (Haagmans *et al*., 2014). Dromedaries can transmit MERS-COVs to humans through direct contact with mucous and nasal secretion or by consuming meat or raw milk (Gossner *et al*., 2016; Mirkena *et al*., 2018). MERS-CoV RNA was detected in milk from milking animals with camels of different origins, as confirmed by qRT-PCR (Reusken *et al*., 2016). Identifying MERS-CoV infection in dromedaries is challenging because of its asymptomatic nature, but experimental cases show tracheitis, bronchitis, rhinitis, nasal discharge, and significant virus release (Haverkamp *et al*., 2018).

From 2010 to 2019, multiple countries confirmed positive BCoV infections in calf feces following the virus's molecular detection using qRT–PCR (Zhu *et al*., 2022). Including Iraq (6.57%) (Mansour*et al*., 2013) and Vietnam (6.9%) (Shin *et al*., 2019), China (18.9.0%) (Keha *et al*., 2019), Thailand (12%) (Singasa *et al*., 2017), India (9.38%) (Kumar *et al*., 2013), Korea (6.2%) (Lee *et al*., 2019), and Korea (5.9%) (Kim *et al*., 2022). In 2002–2003, the prevalence of BCoVs was (33.0%) in Korea and remained between (5.4%) and (15.6%) until 2021 (Jeong *et al*., 2005). This study indicates an increase in the prevalence of BCoVs in Iraq, but it suggests that continuous and potentially harmful infections in bovine farms may persist. This study revealed a (34.6%) prevalence of BCoVs in diarrheal calves on bovine farms, highlighting the need for ongoing epidemiological surveillance to prevent future outbreaks.

This study suggests that the increase in BCoV infections compared with the Iraqi 2013 study and other international studies may be attributable to various factors. First, the studies show a time gap, suggesting that BCoVs developed an evolution strategy to increase their spread during grazing or feedlot bovines' fields. The second reason is the implementation of preventive programs in Western and Asian countries, such as BCoV vaccines, which significantly decrease the incidence of infections.

Research indicates that the virus persists in subclinically infected adult cattle (Park *et al*., 2007; Toftaker *et al*., 2017). BCoV can be shed for up to 14 days through high concentrations of virus particles in respiratory and gastrointestinal secretions (1 billion per milliliter of faeces) (Kapil *et al*., 1990). Moreover, the high density of animals appears to be the primary risk factor for BCoVs (Boileau and Kapil, 2010). Diarrhea in calves is primarily caused by the BCoV virus during the first month of life (Brandão *et al*., 2006; Ammar *et al*., 2014). A study suggests that (10%–30%) of newborn diarrhea cases may be caused by BCoV (Alfieri *et al*., 2018). BCoV is linked to various diseases, including respiratory infections like shipping fever in young cattle and enzootic pneumonia in calves (Saif, 2010).

Environmental studies have shown that animal CoVs remain infectious in water and sewage for up to a year, depending on the temperature and humidity (Mullis *et al*., 2012). Previous studies have shown that the temperature of the surrounding environment significantly influences the stability of CoVs (Siddell *et al*., 1983; Tennant *et al*., 1994). CoVs are inactivated at 56°C for 10–15 minutes, 37°C for a few days and 4°C for a few months. In contrast, CoVs can survive at −60°C for several years without losing their ability to spread (Andries *et al*., 2010; McIntosh *et al*., 1974; Siddell *et al*., 1983). Recent environmental survival research on two animals, CoVs, revealed that they can survive for weeks at 27°C in water and sewage and up to a year at 4°C (Casanova *et al*., 2009).

Conclusion

Betacoronavirus infects camels and bovines and causes diseases in the respiratory and digestive systems of infected animals, leading to their owners incurring significant economic losses. The study revealed that camels have high temperatures, runny noses, and eyes, while bovines have watery diarrhea and sometimes bleeding. According to our findings, the coronavirus spreads quickly on inanimate surfaces and grass, as it remains on these surfaces for long periods. It is recommended that RT-qPCR technology be applied for accurate diagnosis of coronavirus in camels and bovines, emphasizing the importance of managing, following up on, and monitoring the spread of this deadly virus in Iraq.

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Conflict of interest

The authors affirm that there are no conflicts of interest, whether financial, personal, or related to any other relationships, that may affect the integrity of the research presented in this manuscript.

Author contributions

The specific contributions of each author are indicated with initials, i.e. Conception and design of the study: RM and HA. Acquisition of data: IH. Analysis and/or interpretation of the data: HA and IH. Drafting of the manuscript: RM and HA. Critical review/revision: RM and HA.

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