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Molecular and serological incidences of *Bordetella bronchiseptica* in pet dogs with urinary infections

Hadaf Mahdi Kadhim[1], Ahlam Ali Soghi Al-Galebi[1], Mithal K. A. Al-Hassani[1] and Hasanain A. J. Gharban[2]*

[1]*Department of Biology, College of Education, University of Al-Qadisiyah, Al-Diwaniyah, Iraq*
[2]*Department of Internal and Preventive Veterinary Medicine, College of Veterinary Medicine, University of Wasit, Wasit, Iraq*

ABSTRACT

Background: *Bordetella bronchiseptica* is an important bacterial pathogen that is linked primarily to canine infectious respiratory disease complex and rarely to other health issues, including urinary tract infections.

Aims: This study aimed to molecularly identify *B. bronchiseptica* in the urine of diseased dogs with urinary infections and to document local isolates in NCBI. The serological prevalence of anti-*B. bronchiseptica* antibodies in the sera of the study dogs were also assessed.

Methods: A total of 129 pet dogs with urinary tract infections were attended to private clinics in Baghdad province (Iraq) and subjected aseptically to collection of fresh urine and venous blood. Molecular testing of urine samples was performed using the PCR assay, and positive DNA was sequenced, submitted to the NCBI database, and analyzed phylogenetically. Serological testing of sera was performed using ELISA.

Results: Targeting the *16S RNA* gene, PCR assay revealed that 13.92% of study dogs were positive. Phylogenetic analysis of study *B. bronchiseptica* isolates showed the presence of identity with global NCBI-BLAST Chinese *B. bronchiseptica* strain (NCBI-GenBank ID: MT411887.1) at 94.52%–99.66% as the range of similarity and 0.01%–0.0001% as the range of mutation/changes. Serologically, the prevalence rate of anti-*B. bronchiseptica* antibodies by ELISA was 32.91%; in which, the mild, moderate, and severe infections occurring in 53.85%, 34.62%, and 11.54% of cases, respectively. Subsequently, the ODs of mild, moderate, and severe seropositive ODs were 0.560 ± 0.011, 0.686 ± 0.009, and 0.769 ± 0.009, respectively.

Conclusion: This study revealed the prevalence of *B. bronchiseptica* in dogs by molecular testing of urine and serological examination of blood.

Keywords: Canine infectious respiratory disease complex (CIRDIC), Enzyme-linked immunosorbent assay (ELISA), Polymerase chain reaction (PCR), National Center for Biotechnology Information (NCBI), Iraq.

Introduction

Bordetella bronchiseptica is a small, Gram-negative, rod-shaped, coccobacillus bacterium that belongs to the Alcaligenaceae Family in the Burkholderiales Order under the Class of Betaproteibacteria (Bhardwaj, 2013; Lin *et al.*, 2017). Commonly, *B. bronchiseptica* exists within the respiratory tracts of different mammals, such as dogs, with well-known contributions to CIRDIC (Reagan and Sykes, 2020; Reagan, 2021). Humans are an uncommon host to *B. bronchiseptica*; however, exposure to diseased or live-vaccinated animals may increase the chance of infection (Miguelena Chamorro *et al.*, 2023).

The bacterium spreads between dogs either directly *via* licking, nuzzling, coughing, and sneezing, or indirectly by contaminated fomites (Desforges, 2024; Palillo *et al.*, 2024). Worldwide, several reports suggested that the lifecycle of different species of *Bordetella* is significant when it is found inside and/or outside the respiratory tract (Dewan and Harvill, 2019). Briefly, *Bordetella* has several virulence-activated genes (vags) that encode the factors of adhesion (e.g., filamentous hemagglutinin, fimbriae, and pertactin) and enable the adhering of bacteria to ciliated epithelial cells of the respiratory tract. Additionally, the regulatory locus of vags is crucial for expressing virulence factors and controlling their production in response to environmental signals (Mekalanos, 1992). Conversion of genes may potentially initiate the environmental source of frequent unexplained outbreaks caused by different *Bordetella* species (Dewan and Harvill, 2019; Fenwick, 2022; Miguelena Chamorro *et al.*, 2023).

***Corresponding Author:** Hasanain A. J. Gharban. Department of Internal and Preventive Veterinary Medicine, College of Veterinary Medicine, University of Wasit, Wasit, Iraq. Email: *hghirban@uowasit.edu.iq*

In contrast to other bacteria that act as a food source for ameba, *B. bronchiseptica* can evade the predation of ameba, survive within it for a long time, and incorporate and disseminate along with ameba (Ma *et al.*, 2022). Taylor-Mulneix *et al.* (2017) mentioned the capability of *B. bronchiseptica* for growing and disseminating by a complex lifecycle and transferring continuously within a type of ameba known as *Dictyostelium discoideum*. This suggests that the presence of a stable association might permit the bacterium to expand to different geographical environments (Locht *et al.*, 2020; Nugraha *et al.*, 2023). Other researchers have indicated that ameba may act as environmental vectors by which different *Bordetella* species could be expanded and disseminated to infect new mammalian hosts (Rivera, 2020; Samba-Louaka, 2021).

For diagnosis, the sensitivity and specificity of any diagnostic technique are of great importance to enabling the rapid control of infection (Gharban *et al.*, 2023). Although isolation using appropriate media remains the most reliable method, it is usually challenging and requires confirmation using other advanced laboratory techniques (Kadlec and Schwarz, 2018; Ivaska *et al.*, 2022). In recent years, molecular and sequencing tools have considerably enabled the diagnosis of different pathogens (Schmitz *et al.*, 2022; Matys *et al.*, 2024). Therefore, we aimed to identify *B. bronchiseptica* in the urine of diseased dogs with various urinary infections molecularly by PCR assay, phylogenetic analysis of study isolates, and estimating the serological prevalence of anti-*B. bronchiseptica* antibodies in the sera of study dogs by ELISA.

Materials and Methods

Samples

Totally, 79 pet dogs with urinary tract symptoms were attended to private veterinary clinicians in Baghdad province (Iraq) during January (2023) and May (2024) and were subjected to the present study. Under aseptic conditions, fresh urine was collected from each study dog into labeled plastic containers and kept frozen (−20°C) until molecular testing. Additionally, approximately 1.5 ml of venous blood was drained into a free-anticoagulant glass-gel tube and centrifuged at 5,000 rpm for 5 minutes. The obtained sera were pipetted into labeled Eppendorf tubes and kept frozen (−4°C) until serological examination.

Molecular assaying

According to Protocol (A) of the G-Spin™ Total DNA Extraction Kit (Intron, Korea), urine samples were processed, and the DNAs of all study samples were tested using the Nanodrop System (Thermo Scientific, UK) to detect their concentration and purity. Targeting the *16S rRNA* gene, one set of primers was designed [F: (5′- AGC GGT GGA TGA TGT GGT TT-3′) and R: (5′- AGG CTA CCT ACT TCT GGC GA-3′)] based on the NCBI-GenBank *B. bronchiseptica* isolate (JX129161.2), and served to prepare the MasterMix tubes at a final volume of 20 µl. For the PCR reaction, a Thermal Cycler System (Bio Rad, USA) was applied at the following conditions: 1 cycle for initial denaturation (95°C/5 minute), 35 cycles for denaturation (95°C/40 second), annealing (56°C/40 second) and extension (72°C/40 second), and 1 cycle for final extension (72°C/7 minute). For PCR analysis, electrophoresis of agarose gel (1.5%) stained with Ethidium Bromide was conducted at 100 voltage and 80 Am for 90 minutes. The band sizes of the PCR products were visualized under a UV transilluminator (Clinx Science, China), and the positive samples were detected at approximately 504 bp.

Phylogeny

The DNA of all positive *B. bronchiseptica* isolates was sequenced by the Macrogen Company (Korea). The sequence data of the study isolates were submitted to NCBI GenBank to obtain specific access numbers. Then, multiple sequence alignment analysis, phylogenetic tree analysis, and homology sequence identity were performed using MEGA-11 Software and NCBI-Viewer to identify significant identity between the study local *B. bronchiseptica* isolates and the NCBI-BLAST *B. bronchiseptica* isolates/strains.

Serological testing

According to the manufacturer's instructions for the *B. bronchiseptica* antibody ELISA kit (EVL, Spain), the sera in addition to the contents of kits were prepared and processed, and the absorbency values [optical density (OD)] were measured using the Microplate Reader system (BioTek, USA) at 450 nm. The results were then validated and interpreted qualitatively.

Statistical analysis

A one-way ANOVA and *t*-test (GraphPad Prism Software, version 8.0.2) were applied to evaluate significant differences between the study values at $p < 0.05$ (Gharban *et al.*, 2023).

Ethical approval

Approval No. 121/CVM-UQ/18-10-2023 was obtained from the Scientific Committee of the Department of Biology in the College of Education (University of Al-Qadisiyah).

Results

Among the 79 pet dogs, molecular testing of urine samples using the conventional PCR assay revealed that 11 (13.92%) dogs were positive (Figs. 1 and 2). The sequencing data of positive study isolates were submitted to NCBI GenBank at specific access numbers (PQ215813.1, PQ215814.1, PQ215815.1, PQ215816.1, PQ215817.1, PQ215818.1, PQ215819.1, PQ215820.1, PQ215821.1, PQ215822.1, and PQ215823.1). Phylogenetic analysis of the study *B. bronchiseptica* isolates showed the presence of identity with the global NCBI-BLAST Chinese *B. bronchiseptica* strain (NCBI-GenBank ID: MT411887.1) at 94.52%–99.66% as the range of similarity and 0.01%–0.0001% as the range of mutations/changes (Table 1, Figs. 3–7).

Serological examination of 79 pet dogs by ELISA revealed 26 (32.91%) positive samples; in which, the mild, moderate, and severe infections were observed in 14 (53.85%), 9 (34.62%), and 3 (11.54%), respectively. Subsequently, the ODs of the mild, moderate, and severe seropositive groups were 0.560 ± 0.011, 0.686 ± 0.009, and 0.769 ± 0.009, respectively (Figs. 8–10).

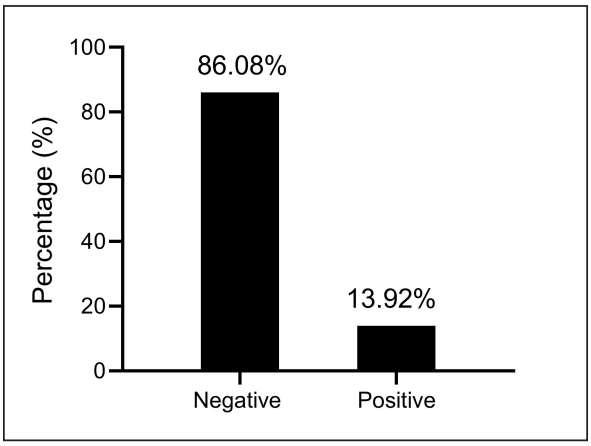


Fig. 1. Total results for testing urine samples by molecular PCR.

Discussion

Bacterial infections represent a major obstacle to public health, linked to several morbidities and mortality in animals and humans. Although great advancements have been made in preventive and therapeutic measures, bacteria remain a cause of great economic losses and social burdens (Gharban and Yousif, 2020; Janik *et al.*, 2020; Mestrovic *et al.*, 2022). In the present study, the molecular and serological data indicated that the prevalence rates of *B. bronchiseptica* were 13.92% and 32.91%, respectively. In comparison to other worldwide studies, the percentages were 7.4% in Japan (Mochizuki *et al.*, 2008), 45.6% in Germany (Schulz *et al.*, 2014), 1.94% in India (Rose *et al.*, 2024), and 15.47%–36.23% in China (Shang *et al.*, 2024). This variation

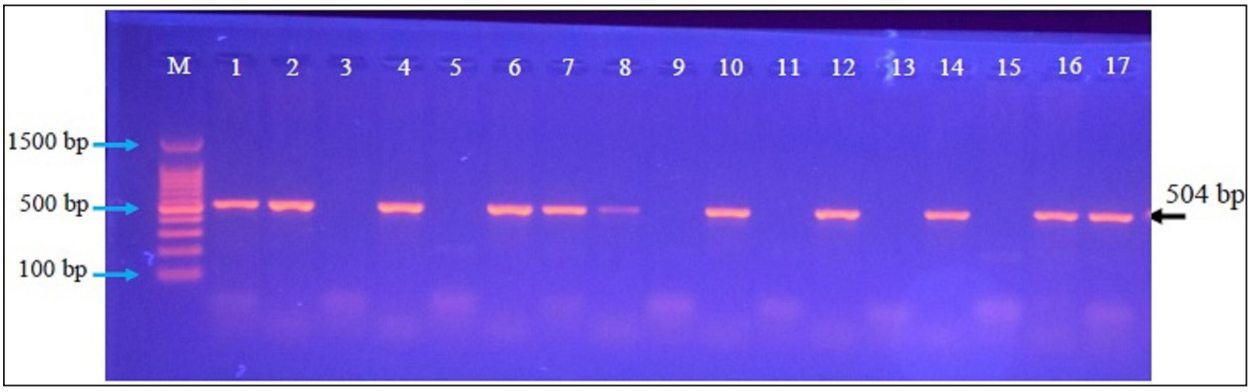


Fig. 2. Agarose-gel electrophoresis of PCR products at 100 Volt and 80 Am for 90 minutes. M: Ladder marker (100–1,500 bp); Lanes (3, 5, 9, 11, 13, and 15): Negative samples; Lanes (1, 2, 4, 6, 7, 8, 10, 12, 14, 16, and 17): Positive samples at ≈ 504bp.

Table 1. Homology sequence identity (%) of local and NCBI-BLAST *B. bronchiseptica* isolates.

Local isolate		NCBI-BLAST isolate				
Name	**Access no.**	**Species**	**Country**	**Host**	**Access no.**	**%**
Iraq-Pet Dog 1	PQ215813.1	*B. bronchiseptica*	China	Cat	MT411887.1	97.46
Iraq-Pet Dog 2	PQ215814.1	*B. bronchiseptica*	China	Cat	MT411887.1	98.7
Iraq-Pet Dog 3	PQ215815.1	*B. bronchiseptica*	China	Cat	MT411887.1	97.44
Iraq-Pet Dog 4	PQ215816.1	*B. bronchiseptica*	China	Cat	MT411887.1	98.52
Iraq-Pet Dog 5	PQ215817.1	*B. bronchiseptica*	China	Cat	MT411887.1	99.66
Iraq-Pet Dog 6	PQ215818.1	*B. bronchiseptica*	China	Cat	MT411887.1	98.34
Iraq-Pet Dog 7	PQ215819.1	*B. bronchiseptica*	China	Cat	MT411887.1	96.02
Iraq-Pet Dog 8	PQ215820.1	*B. bronchiseptica*	China	Cat	MT411887.1	94.52
Iraq-Pet Dog 9	PQ215821.1	*B. bronchiseptica*	China	Cat	MT411887.1	95.93
Iraq-Pet Dog 10	PQ215822.1	*B. bronchiseptica*	China	Cat	MT411887.1	95.91
Iraq-Pet Dog 11	PQ215823.1	*B. bronchiseptica*	China	Cat	MT411887.1	95.38

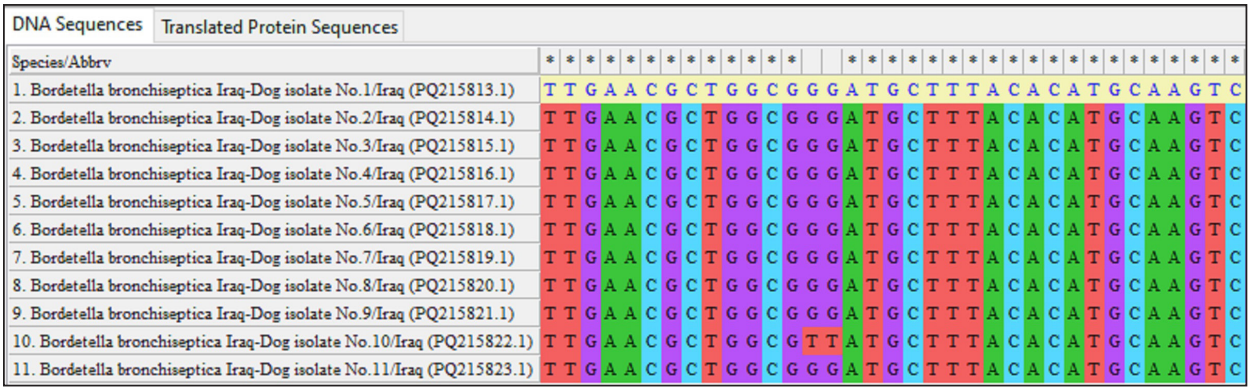


Fig. 3. Multiple sequence alignment of local *B. bronchiseptica* isolates using MEGA-11 software.

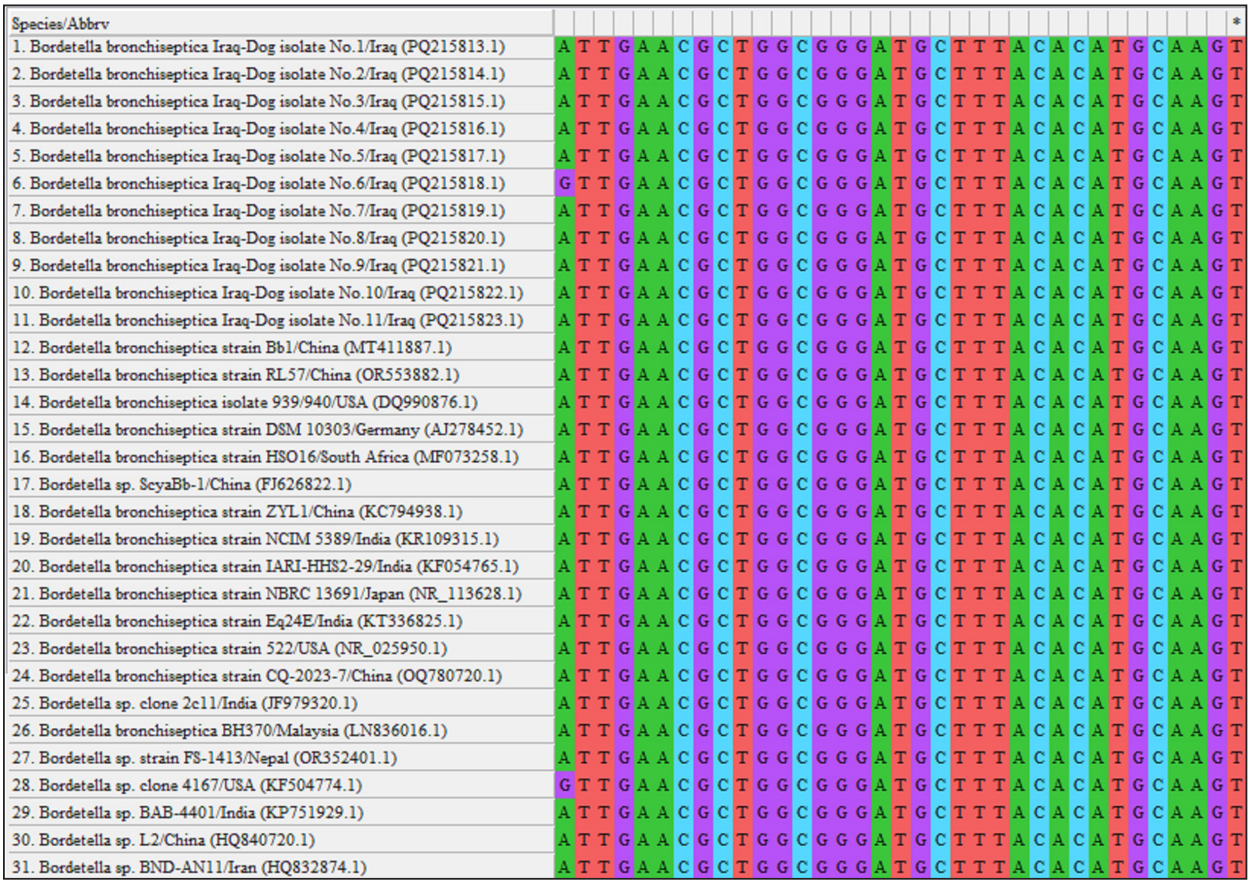


Fig. 4. Multiple sequence alignment of local *B. bronchiseptica* isolates and NCBI-BLAST *B. bronchiseptica* isolates/strains using MEGA-11 software.

in the prevalence of *B. bronchiseptica* infection might be attributed to several factors, including demographic characteristics, environmental conditions, and health care practices. Although traditional diagnostic techniques based on culture and isolation remains the gold standard, they are difficult to perform, interpret, and are time-consuming. Therefore, many serological techniques have been used for the rapid assessment of *B. bronchiseptica* in various animals and humans (Di Bonaventura *et al.*, 2021; Zhang *et al.*, 2021; Calderaro *et al.*, 2022). During outbreaks, although these techniques are widely used as fast and simple tools for detecting different infections, they have a reduced degree of specificity by yielding false-positive results

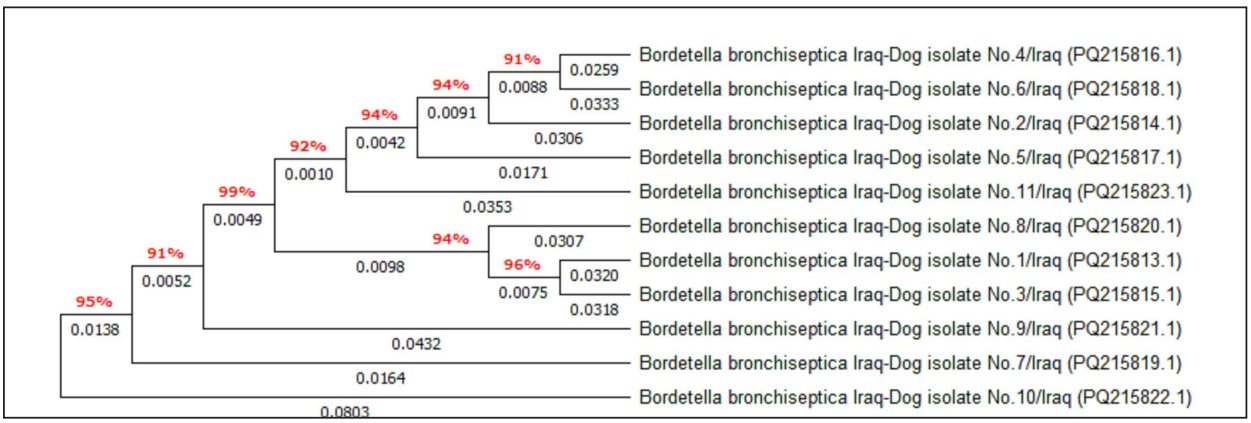


Fig. 5. Phylogenetic tree analysis of local *B. bronchiseptica* isolates using MEGA-11 software.

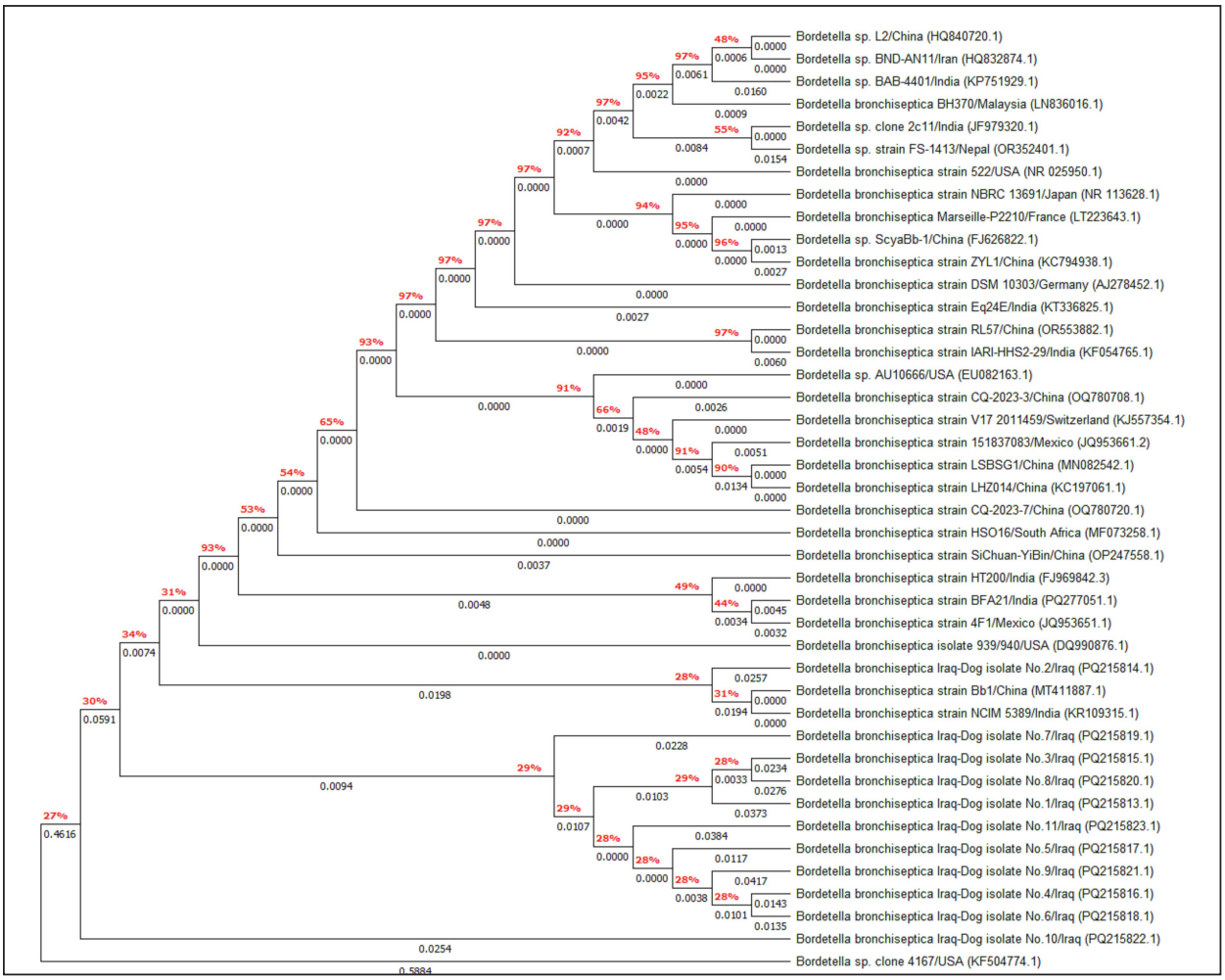


Fig. 6. Phylogenetic tree analysis of local *B. bronchiseptica* and NCBI-BLAST *B. bronchiseptica* isolates/strains using MEGA-11 software.

due to cross-reaction and past exposure (Al-Graibawi *et al.*, 2021; Smolejová *et al.*, 2023). For this reason, PCR exploits to sustaining the accurate and rapid identification of *B. bronchiseptica* in different clinical specimens by targeting genus- and species-specific genes (Fastrès *et al.*, 2020; Hashish *et al.*, 2021; Cole

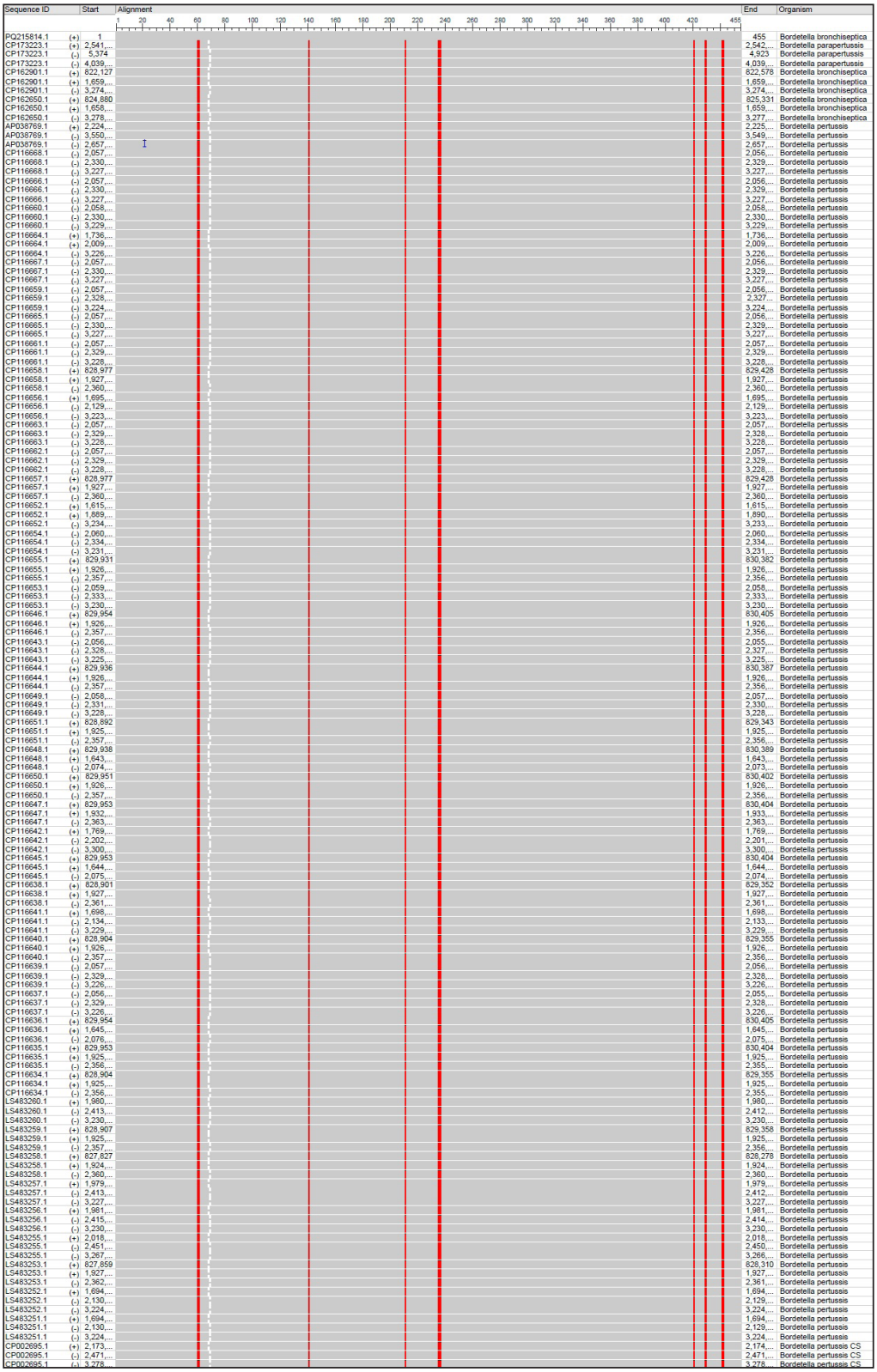


Fig. 7. Multiple sequence alignment of local and NCBI-BLAST *B. bronchiseptica* isolates/strains using the NCBI MSA Viewer.

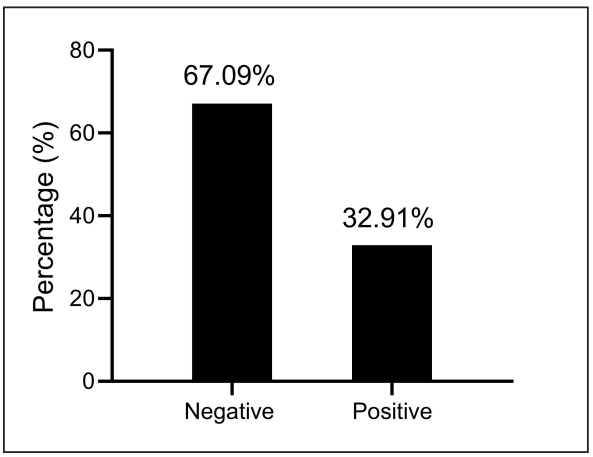
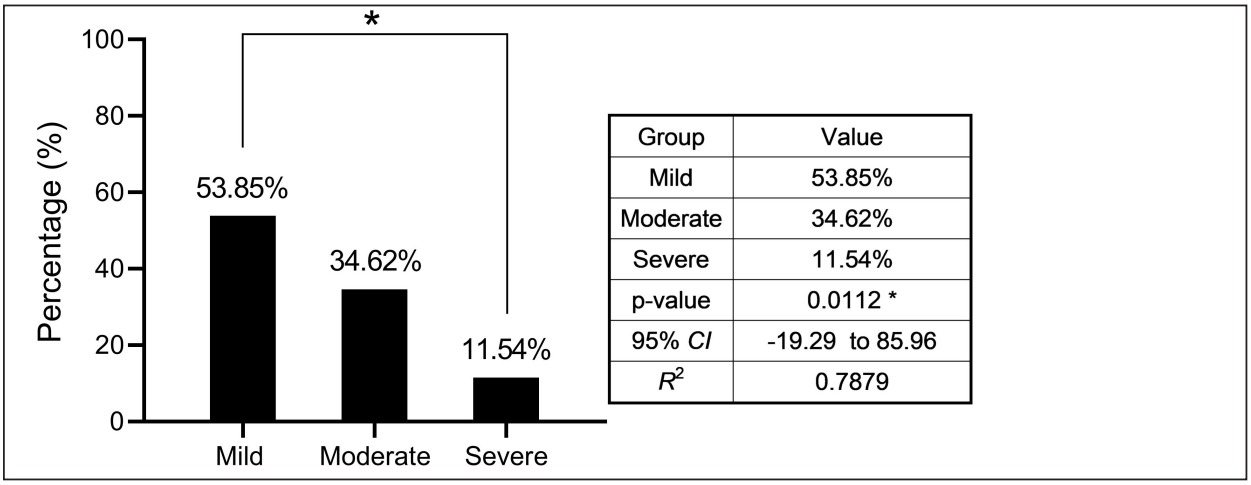


Fig. 8. Total results for testing the serum samples by serological ELISA.

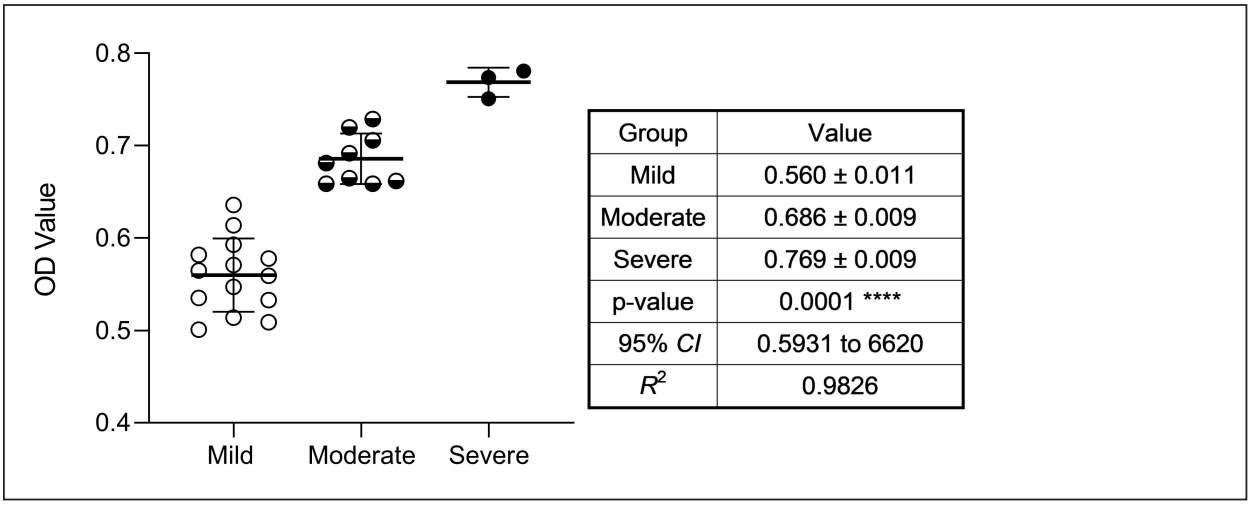
et al., 2024). When both detection methods, ELISA and PCR, were compared regarding their ability to detect *B. bronchiseptica* in dogs, PCR was found to be more sensitive than ELISA in the detection of *B. bronchiseptica* (Batrinou *et al.*, 2022; Bhardwaj *et al.*, 2024). The high sensitivity and specificity of PCR may be attributable to its ability to amplify small amounts of DNA, with detection even at low copy numbers for target sequences (Palka-Santini *et al.*, 2009; Mirabile *et al.*, 2024). Additionally, PCR targeting specific DNA sequences can lead to high specificity by differentiating between closely related bacteria or genetic variants (Leonardo *et al.*, 2021; Smolejová *et al.*, 2023).

Phylogenetic analyses have already been combined with the data of various researchers to evaluate genetic relatedness among canine *B. bronchiseptica* isolates (Nicholson and Shore, 2024). Targeting the *16S rRNA* gene, phylogenetic analysis of *B. bronchiseptica*



Group	Value
Mild	53.85%
Moderate	34.62%
Severe	11.54%
p-value	0.0112 *
95% *CI*	-19.29 to 85.96
R^2	0.7879

Fig. 9. Prevalence rate (%) of mild, moderate, and severe seropositive infections according to the severity of OD.



Group	Value
Mild	0.560 ± 0.011
Moderate	0.686 ± 0.009
Severe	0.769 ± 0.009
p-value	0.0001 ****
95% *CI*	0.5931 to 6620
R^2	0.9826

Fig. 10. Values (Mean ± Standard error) of seropositive ODs according to severity.

isolates demonstrated their identity to the Chinese NCBI-BLAST *B. bronchiseptica* strain. In bacteria, the *16S rRNA* gene encodes the small subunit ribosomal RNA molecules of ribosomes, which are responsible for the essential process of converting genetic messages to functional cell components *via* the translation of mRNA to proteins (Chiaruttini and Guillier, 2020; Gong and Koudelka, 2023). Since the advent of high-throughput sequencing, PCR-amplified *16S rRNA* genes have typically been clustered based on their similarity to generate operational taxonomic units (OTUs), and then, representing the OTU sequences compared with the reference databases to infer likely taxonomy (Yeh *et al.*, 2021; Egenriether *et al.*, 2022).

Conclusion

This may represent the first Iraqi study to elucidate the prevalence rate of *B. bronchiseptica* in dogs. Molecular PCR testing of urine and serological identification of anti-*B. bronchiseptica* antibodies by ELISA confirm the circulation of infection in study dogs and their areas. Phylogenetic analysis of isolates targeting the *16S rRNA* gene provides important information about the possible source of infection. However, further studies are necessary to estimate the prevalence rate of infection in other local areas and to demonstrate the existence of other *Bordetella* species in various livestock and wildlife animals.

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Conflict of interest

No.

Funding

No funds were received to complete this work.

Authors' contribution

HMK: Molecular testing of urine samples. AASA: Serological testing of serum samples. MKAA: Collection of urine and blood samples. HAJG: Phylogenetic and statistical analyses of obtained data. All authors contributed to study design and manuscript writing.

Data availability

The obtained data were included in this manuscript.

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