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Dogs

Investigation of the Correlation Between ELISA and Serum Amyloid A in the Diagnosis of *Bordetella bronchiseptica* in Dogs

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ABSTRACT

Background: *Bordetella bronchiseptica* is an essential bacterial pathogen characterized by chronic respiratory disease in dogs known as Kennel cough. The presence of causative antibodies in animals can also be detected by lipopolysaccharide antigen-based enzyme linked immunosorbent assay (ELISA). In recent years, it has been determined that there is a significant relationship between acute phase proteins and diseases, and disease follow-up can be done within the framework of this relationship.

Methods: In this study, blood sera from 150 dogs in an animal shelter in Van province were evaluated for *B. bronchiseptica* by the homemade ELISA method, and their correlations with serum amyloid A (SAA) were investigated. Blood serum samples were analysed for antibodies against *B. bronchiseptica* using a homemade ELISA method. Positive animals were also molecularly confirmed using nasal swabs by PCR. A commercial ELISA kit determined SAA levels in blood sera.

Results: Eighteen (12%) of the analysed blood serum samples were found positive by the homemade ELISA method. SAA concentrations in the positive blood sera were elevated from 12.7 to ≤38.98 mg/L. SAA concentrations in blood sera serologically positive for *B. bronchiseptica* were statistically significant.

Conclusions: In this study, in which the relationship between SAA concentration and *B. bronchiseptica* was investigated for the first time in Turkey, it was concluded that SAA concentration analysis may help diagnose and monitor the disease. In addition, the presence and prevalence of this critical and zoonotic agent causing chronic respiratory tract disease in dogs in Van province was revealed for the first time in this study.

1 | Introduction

Kennel cough (Canine Infection Respiratory Disease—CIRD) is defined as a complex respiratory disease caused by the synergistic effect of multiple agents. The entry of pathogens into kennels and intensively housed animal shelters causes respiratory problems that are frequently observed. CIRD outbreaks are pretty common and more severe in shelters (Decaro et al. 2016). It is known that canine adenovirus 2, canine distemper virus, canine herpesvirus and canine parainfluenza virus type 2, and the most important bacterial pathogens, *B. bronchiseptica*, are among the viral pathogens commonly seen in CIRD cases (Cordisco et al. 2022).

Summary

- This is the first study in Turkey investigating the correlation between ELISA and SAA in diagnosing *B. bronchiseptica* in dogs.
- This is the first prevalence study of *B. bronchiseptica* in dogs in Van (Eastern Türkiye) and its region.
- It is a supportive study to include SAA analysis among the routine analyses in diagnosing infectious diseases.

It is stated that *Bordetella* agents causing bordetellosis are composed of 17 species belonging to the Alcaligenaceae family under the *Betaproteobacteria* class (Ivanov et al. 2016). *Bordetella* species have a small, Gram-negative, aerobic and coccobacillary morphology (Lorenzo-Pajuelo et al. 2002). The classical *Bordetella* pathogens *B. pertussis*, *Bordetella bronchiseptica* and *Bordetella parapertussis* are characterized by respiratory tract infections in mammals, whereas other species are associated with respiratory infections in different animal species and humans, as well as some other diseases (Badhai and Das 2023).

B. bronchiseptica is a vital pathogen causing fatal chronic respiratory infections in dogs with symptoms of coughing, sneezing, enlarged lymph and severe pneumonia. The disease is recognized as a significant threat in many hosts, such as rabbits, pigs, dogs and guinea pigs, which are complex and time-consuming to treat (Hosseindoust et al. 2023; Li et al. 2023). *B. bronchiseptica* rarely causes infections in immunocompromised people. The fact is that *B. bronchiseptica* is often accompanied by other microorganisms during the isolation and identification of *B. bronchiseptica* by classical culture method creates significant difficulties in diagnosing the disease. On the other hand, it is also known to be a factor that prepares the ground for secondary infections in the host's colonization process (Rybolt et al. 2022). It has been determined that people with immunocompromised conditions such as HIV virus, lung cancer after chemotherapy, rheumatoid arthritis, cystic fibrosis and haematological malignancies are also infected by *B. bronchiseptica* (Gomez et al. 1998; Monti et al. 2017). However, although the epidemiological modes of transmission of *B. bronchiseptica* are still unclear, it has been reported that people in close contact with their pets are at high risk for asymptomatic colonization or infection via the aerosol route and environmental reservoir route and that the risk increases even more after contact with sick dogs or cats diagnosed with *B. bronchiseptica* (Yacoub et al. 2014; Monti et al. 2017; Badhai and Das 2023). In a study conducted in Canada, the agent was investigated by serological tests in polar bears (24%), and despite the prevalence of the disease in domestic dogs, no correlation was found between the possibility of transmission from dogs, and it was emphasized that infections could be transmitted by aerosol route or by close contact of polar bears (Bryan et al. 2011; Pilfold et al. 2021). Vaccines against *B. bronchiseptica* for cats and dogs do not provide lifelong protection, and revaccination is necessary (Day et al. 2016). Intranasal vaccine forms have been reported to be highly effective (Chamorro et al. 2023).

Acute phase reaction is considered a process involving metabolic changes in the nonspecific defence mechanism of the host exposed to a pathogenic agent. These reactions are reported to start as a local reaction induced by cytokines synthesized by activated macrophages (Paltrinieri 2008). During an acute phase reaction, the concentration of specific proteins in the blood changes dramatically due to a complex induction pattern involving cytokines and glucocorticoids. These proteins are called 'acute phase proteins.' Serum amyloid A (SAA) is thought to function in the regulation of lipid metabolism, lipid transport, chemotaxis and the inflammatory process in epithelial cells from various normal tissues such as the brain of Alzheimer's disease patients, breast lobules in sick people, colon mucosa, prostate, kidney and lung (Upragarin et al. 2005). Although a physical examination of six healthy cats experimentally infected with *B. bronchiseptica* in Japan showed no signs of bronchopneumonia, a slight increase in SAA values in blood sera was found. In addition, an early increase in SAA serum concentrations was observed in infected animals (Shida et al. 2012).

In this study, *B. bronchiseptica* antibodies were analysed in blood serum samples obtained from dogs using a homemade enzyme linked immunosorbent assay (ELISA) method, and SAA concentrations were compared with positive samples to determine their correlation.

2 | Materials

2.1 | Nasal Fluid and Serum Samples

A total of 150 blood and 150 nasal swab samples were taken from dogs undergoing sterilization operations in the Animal Care and Rehabilitation Centre of the Metropolitan Municipality of Van Province and delivered to the laboratory of the Department of Microbiology, Faculty of Veterinary Medicine, Van Yuzuncu Yil University under the cold condition. Blood samples were centrifuged at 2000 *g* for 10 min, serum was separated, and all samples were stored at −80°C until use.

2.2 | Reference Culture

The reference culture of *B. bronchiseptica* (ATCC 4617) was used to prepare the antigen for the homemade ELISA test.

3 | Methods

3.1 | *B. bronchiseptica* Homemade ELISA Test

3.1.1 | Antigen Preparation

The presence of antibodies against *B. bronchiseptica* in blood serum samples taken from dogs was determined by homemade ELISA. *B. bronchiseptica* culture to be used to prepare ELISA antigen was inoculated in BHI medium containing 0.1% Tween 80 and incubated at 37°C for 72 h. The liquid culture was centrifuged at 8000 rpm for 30 min, and the pellet was washed three times with PBS. 0.05% merthiolate and 0.03% formaldehyde (30%) were added as a preservative. The inactivated antigen was sonicated, and its density was adjusted to 1.5×10^6 cfu/mL and used as bacterial ELISA antigen. Protein amounts of the

bacteria were determined with a BIORAD protein measurement kit (Bradford 1976).

3.1.2 | Antigen Coating

For the preparation of ELISA plates, *B. bronchiseptica* whole-cell antigens and positive and negative control sera were used, and ideal antigen, serum and conjugate dilutions were determined by the chequerboard method. Then, whole cell antigens were diluted with coating buffer (Carbonate/bicarbonate, pH 9.6), and ELISA plates were coated. The 100 µL of antigen reconstituted with carbonate/bicarbonate buffer system was placed in the wells of the flat-bottomed ELISA plate and incubated at 37°C for 1 h and then at +4°C for one night. Antigen-coated plates were washed three times with PBS-T (0.15 mol/L PBS, %0.5 Tween 20, pH 7.2).

3.1.3 | Indirect ELISA Procedure

Detection of *B. bronchiseptica* antibody was conducted by ELISA protocol. For this purpose, 100 µL of 10-fold diluted serum samples was placed in each microplate well and incubated at 37°C for 1 h. After washing the plates three times with PBS-T, 100 µL horseradish peroxidase-labelled rabbit anti-dog IgG (Sigma, A 6792) was added and incubated at 37°C for 30 min. After washing, 100 µL of 0.4 mg/mL σ-phenylenediamine dihydrochloride substrate (Sigma, P8287) was added to all wells and incubated at room temperature for 20 min and plates were read at 450 nm in an ELISA reader (Antos, htII, Austria). The negative threshold value was calculated to evaluate ELISA results as the arithmetic mean + 3-fold standard deviation (NKORT +3 SD) of the OD values of 20 control sera.

3.2 | Multiplex PCR

Genomic Mini isolation kit (A&A Biotechnology, Poland) was used for DNA isolation from swab samples, and DNA samples were stored at −20°C until analysis. For molecular confirmation of *B. bronchiseptica*, 16S rRNA F and genus-specific Fla1 + Fla2 primer sets targeting the highly conserved 16S rRNA region were used (Table 1).

A total of 1.5 µL from each primer pairs, 12 µL of Master Mix, 5 µL of DNA and 3.5 µL of sterile ultrapure water were prepared for a total of 25 µL for amplification in each PCR tube content. This mixture was amplified in a thermal cycler with a 35-cycle reaction, each cycle consisting of 15 s at 95°C, 30 s at 53°C and 30 s at 72°C, after 5 min of pre-denaturation at 95°C. As a final extension, after the DNAs were kept for 5 min at 72°C (Hozbor et al. 1999; Register and Yersin 2005), 1.5% agarose gel containing 5 µg/mL ethidium bromide was prepared and subjected to electrophoresis for imaging.

3.3 | SAA ELISA Test

An ELISA kit (Multispecies SAA ELISA kit, Tridelta Development Ltd., Kildare, Ireland) was used to determine SAA levels in blood sera obtained from dogs. All sera were analysed in duplicate according to the manufacturer's recommendation. Final absorption was read at 450 and 630 nm in an ELX 800 ELISA Microplate Spectrophotometer (Bio-Tek Instruments Inc., USA).

3.4 | Statistical Analyses

Descriptive statistics are given as mean and standard error. Statistical analyses of the samples obtained were performed by independent two-sample *t*-test with a confidence interval of $p < 0.01$. The correlation between ELISA test results and SAA was evaluated by the Pearson Correlation test. ROC curve analysis was used to determine whether SAA is an indicator for *B. bronchiseptica* disease.

4 | Results

4.1 | PCR

To verify the presence of *B. bronchiseptica*, PCR testing was conducted on swab samples collected from ELISA-positive animals. The PCR results confirmed that all ELISA-positive samples were indeed positive for *B. bronchiseptica*. The 16s rRNA, fla1/fla2 and fla3/fla4 gene regions of the positive samples were further confirmed by PCR (Figure 1).

4.2 | *B. bronchiseptica* ELISA Results

Of the total of 150 serum samples, 18 (12%) were positive for *B. bronchiseptica* by ELISA. The negative threshold value was determined as 0.9 for evaluating the ELISA results obtained. The

TABLE 1 | PCR primers used in the study.

Name of primers	Primer sequences 5'-3'	Amplicon size	Literature
fla1F	5'-CCCCCGCACATTTCCGAACTTC-3'	237 bp	Hozbor et al. (1999)
fla2R	5' AGGCTCCCAAGAGAGAAAGGCTT-3'		
fla3F	5'-CCCCCGCACATTTCCGAACTTC-3'	164 bp	Hozbor et al. (1999)
fla4R	5' AGGCTCCCAAGAGAGAAAGGCTT-3'		
16S rRNA F	5' AGAGTTTGATCCTGGCTCAG -3'	520 bp	Register and DeJong (2006)
16S rRNA R	5' GCGGCTGCTGGCACG -3'		

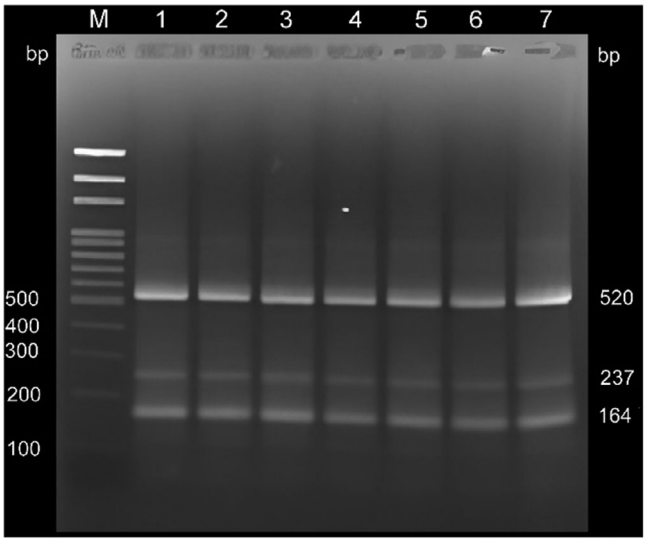

FIGURE 1 | A agarose gel electrophoresis of gene PCR products of *Bordetella bronchiseptica* isolates. Lane 1:100 bp marker (Sigma, P1473); lane 2–8: *of B. bronchiseptica* positive field strains (fla1; 164 bp -fla2: 237 bp, 16S rRNA: 520 bp).

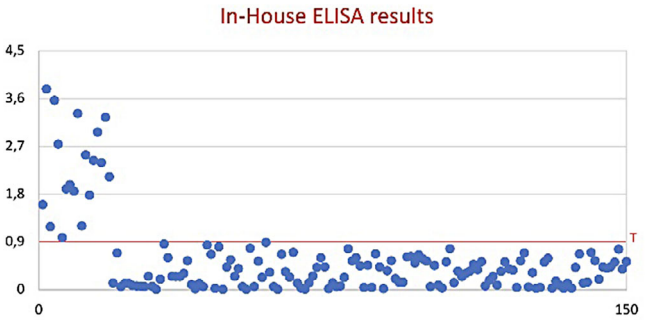

FIGURE 2 | Antibody levels of positive and negative samples for *Bordetella Bronchiseptica*. (Independent 2-sample T-test).

antibody level in the blood serum of the samples confirmed by PCR (Group A) was observed to be between 0.985 and 3.78. ELISA results of negative samples (Group B) were between 0.02 and 0.78 (Figure 2).

4.3 | SAA Concentrations

In Group A, which was determined to be positive for *B. bronchiseptica*, SAA concentrations ranged between 12.7 and ≤38.98 mg/L (Figure 3). SAA concentrations in Bordetella-negative samples ranged between 0.002 and ≤0.86 mg/L. Accordingly, the mean SAA in ELISA-positive samples was 31.65, while the mean SAA in ELISA-negative samples was 10.02. ELISA positive samples had three times more SAA concentration than negative samples (Table 2).

When SAA concentrations in ELISA-positive and negative samples were evaluated, it was determined that ELISA-negative samples had a significant positive correlation with Pearson Correlation (P<0.01) statistical analysis (Figure 4a). No statistically significant correlation was found in ELISA-positive samples

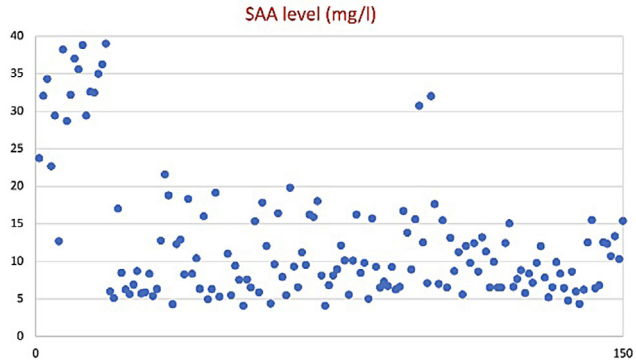

FIGURE 3 | SAA concentrations of positive and negative samples for *B. bronchiseptica* (Independent 2-sample T-test).

TABLE 2 | T-test results for ELISA and SAA positive and negative samples

Tests	Results	Mean	Std. Error Mean	*p*
ELISA	Negative	0,32	0.02	<0,001
	Positive	2,31	0.20	
SAA	Negative	10,02	0.42	<0,001
	Positive	31,65	1.57	

(Figure 4b) (Table 3). According to the ROC curve analysis, the diagnostic performance of SAA concentration was statistically significant (Figure 5). The AUC value was determined as 0.983 and this value was found to be very close to 1. The cut off value for SAA concentration was determined as 22.125. When SAA concentrations exceed this value, it can be concluded that the disease is positive. The sensitivity and specificity for the cut-off value was 94.4% and 98.5%, respectively (Table 4).

5 | Discussion

B. bronchiseptica is one of the causative agents of respiratory tract infections in dogs. Although it is the primary causative agent of kennel cough in dogs in crowded shelters, it commonly causes respiratory tract infections in various animal species (Chalker et al. 2003). It is known that where there are large numbers of stray and uncontrolled dog herds, such as in large populated areas, humans and other animals are threatened by zoonosis and infectious infections (Sayin et al. 2016). The isolation of *B. bronchiseptica* from a person with a severe respiratory tract infection and a history of severe cough in his dog demonstrate the zoonotic importance of the disease (Tatem et al. 2023).

Different studies show the presence of *B. bronchiseptica*. For example, some studies have reported that female animals are more susceptible to the disease or that it is observed more frequently in the summer months. More comprehensive studies are needed to determine the transmission potential of the agent and to understand how it is protected in defence systems and how it spreads (Pilfold et al. 2021). Some studies have confirmed the

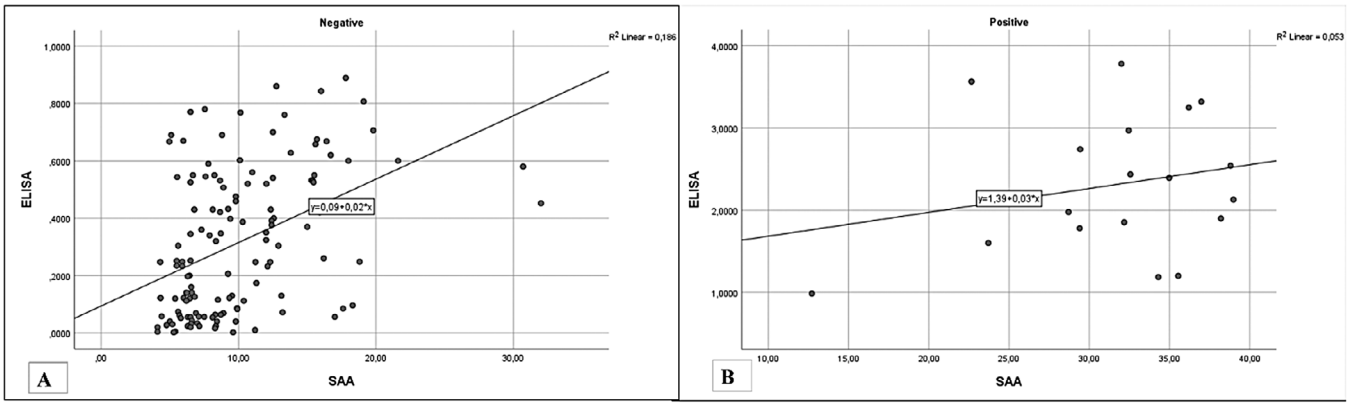

FIGURE 4 | Correlation between ELISA test results and SAA results. (A- The relationship between ELISA negative and SAA concentrations, B- The relationship between ELISA positive and SAA concentrations).

TABLE 3 | Correlation Coefficients between Positive and Negative ELISA and SAA Concentration.

		SAA
ELISA	Negative	0,432**
	Positive	0.231

**Correlation is significant at the 0.01 level (2-tailed). Pearson Correlation test

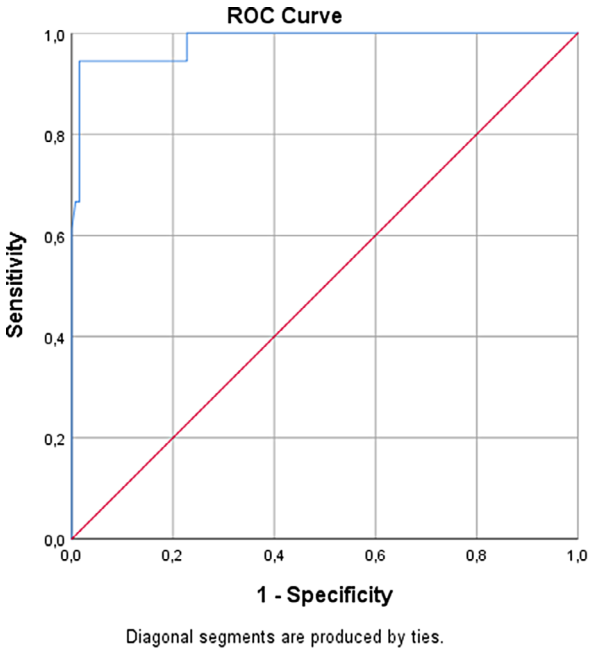

FIGURE 5 | ROC Curve of cut-off values for SAA Concentrations.

presence of *B. bronchiseptica* in a large proportion of dogs with no clinical symptoms (Chalker et al. 2003).

A study conducted in two different kennels with 40 cats and 40 dogs in the United States reported that the disease was frequently isolated during periods of coughing, and the prevalence may decrease during periods without coughing. Accordingly, it was found to be 47% in cats and 23% in dogs during the cough period. The same study emphasized that cats are not crucial in

TABLE 4 | Results of ROC Curve Analysis.

AUC	Std. Error[a]	Cut Off	*p*	95% Confidence Interval	
0.983	0.013	22.125	0.000	0.958	1.000

Abbreviation: AUC, Area Under the Curve.

the pathogenic role of *B. bronchiseptica* by advanced molecular methods (Foley et al. 2002). Earlier studies have documented the prevalence of *B. bronchiseptica* in cats, with a prevalence of 85% in the United Kingdom and 30% in healthy cats (McArdle et al. 1994).

The disease was investigated by molecular methods in 35 domestic cats in Poland, and 16 of them were found to be positive for the respiratory tract. The study emphasized the need for accurate disease diagnosis in terms of disease control. In addition, it was stated which *Bordetella* genotypes are the most common in cats and dogs, and the preparation of the appropriate vaccine is important (Garbal et al. 2016). In Italy, 138 dogs were screened for CIRD by RT-PCR, and 9 (6.51%) *B. bronchiseptica* were detected. It was concluded that the prevalence of viral and bacterial CIRD agents has increased; therefore, specific vaccines are needed. In addition, it is emphasized that the epidemiological status of the disease according to geographical regions should be determined, and different vaccine formulations should be prepared according to different countries (Decaro et al. 2016). In a comprehensive CIRD study of 503 asymptomatic dogs in the United States, *B. bronchiseptica* was found to be 40.8%. The study also pointed out that reinforcing factors such as environmental stress, impaired immune function, nutritional deficiencies or the coexistence of other pathogens can lead to primary or secondary infections in asymptomatic dogs. Therefore, the importance of vaccinating the animals when they are brought to shelters, shortening the duration of their stay, and providing shelter conditions that minimize stress and contact was emphasized (Lavan and Knesl 2015).

In a study conducted in Canada in which 102 dogs were tested using the ELISA method, disease prevalence was detected in 65% of the dogs. It was emphasized that *B. bronchiseptica* was likely to be endemic in dogs in the tested region in BC. It was

also reported that the disease may cause mild symptoms alone but may cause severe symptoms when associated with other pathogens (Bryan et al. 2011). In a study on respiratory tract pathogens in New Zealand dogs, the presence of *B. bronchiseptica* was determined as positive in 3 animals (6%) by quantitative PCR (qPCR) method from samples taken from 47 healthy animals (Sowman et al. 2018). In a study conducted in Konya, Turkey, 96 (48.7%) positive samples in 196 animals were detected by PCR and standard microbiological methods (Sayin et al. 2016). In addition, *B. bronchiseptica* was isolated from 8 (61%) of 13 diseased shelter dogs and 10 (8.4%) of 119 healthy shelter dogs in Turkey (Erdeğer 2002). In this study, 12% of the 150 blood serum samples analysed were positive for *B. bronchiseptica*. Compared to other studies, *B. bronchiseptica* was found to be at a lower rate in stray dogs living in open areas in the region. This situation was related to the geographical aspect and the presence of dogs in inaccessible or closed areas.

APPs have played an essential role in the clinical setting in recent years, from diagnosis to prognosis and treatment monitoring. They have been reported to be valuable markers to detect the presence or absence of various diseases in cats. Measurement of APPs in cats with feline infectious peritonitis (FIP) may help differentiate between FIP and non-FIP cats with similar clinical signs (Rossi 2023). SAA's have been measured as APPs in various diseases, and their relationship with the disease has been determined (Shida et al. 2012). In a study in Turkey, SAA levels of bovine abortion cases were measured, and it was reported that it may be a new marker that can be used in diagnosis (Aras and Yavuz 2022). In this study, determining the prevalence of infections such as *B. bronchiseptica* infections, especially rabies, in the Van region, where there are many uncontrolled stray animals, is extremely important for controlling and combating the disease. For this purpose, blood sera were collected from shelter dogs from the animal shelter of Van province in 2022, and serological studies were carried out on the disease.

To investigate the effects of SAA in bronchopneumonia, 17 cats were vaccinated against *B. bronchiseptica*, and antibody levels were measured and evaluated with SAA concentrations. In the study, *the B. bronchiseptica* titter of the vaccine was determined in cats, but bronchopneumonia could not be detected clinically. However, they proved that SAA values increased (Shida et al. 2012). The use of cats in the study and the fact that *B. bronchiseptica*, the causative agent of pneumonia, could not be detected in dogs differed from this study. However, the increase in SAA concentration can be considered a common finding in our study. In a study conducted in pigs in Poland, SAA was correlated with the presence of the Influenza virus and *Bordetella* bacteria, which are the causative agents of respiratory diseases, and it was emphasized that SAA could be used in respiratory diseases in accordance with our study. In addition, it was shared that the markers used in the study reflect the severity of the disease and may enable the course of the disease, rapid diagnosis and appropriate treatment (Pomorska-Mól et al. 2015). In another study, which is compatible with our study, the APPs (C-reactive protein [CRP] and SAA) correlations with the disease were measured in 19 dogs diagnosed with Bacterial Pneumonia and 64 healthy dogs in Helsinki. In this study, APP ratios in diseased dogs significantly increased compared to healthy dogs. The study observed that CRP and SAA levels decreased rapidly after treating sick dogs, and it was emphasized that these markers are valuable markers in observing treatment response. In addition, it was emphasized that the concentration range of SAA is more prominent than CRP due to its wider range and that it is a better diagnostic and follow-up marker (Viitanen et al. 2017). In another study similar to ours, it was found that there was a significant correlation between CRP concentration, known as APP, and disease in dogs with *B. bronchiseptica* infection in France and Belgium. The same study emphasized that it would be more meaningful to measure SAA instead of CRP in dogs with bacterial pneumonia (Canonne et al. 2021). There are different studies on SAA concentrations. To characterize SAA concentrations during diagnosis and treatment in cats with lymphoma, 16 cats with lymphoma and 25 healthy cats were tested. SAA concentrations were higher in cats with lymphoma at diagnosis time than in healthy cats. It was reported that SAA may be helpful to protein markers for monitoring antineoplastic therapy in cats with lymphoma (Winkel et al. 2015). Another study found that SAA concentrations in 17 cats with inflammation or tumours were approximately 30 times higher than in normal cats. They also concluded that the SAA test in cats is also practical for patients exhibiting jaundice, haemolysis and chylemia (Ishioka and Hayakawa 2019). In another study, 26 dogs with pyometra and 18 healthy female dogs were tested through APPs, and it was found that the concentration was significantly higher in animals with pyometra, and it was concluded that it was related to the disease (Hagman 2011). Our study evaluated the findings obtained in all these studies. This study used the ELISA method to analyse the presence of SAA in blood serum samples taken from stray dogs living free in Van and its region. Although the presence of SAA was significantly increased in serologically positive samples for *B. bronchiseptica*, the presence of SAA was found to be low in negative cases. As mentioned above regarding APPs, especially SAA has emerged as important biomarkers in the detection and monitoring of various infectious diseases. The rapid increase in SAA levels during inflammatory responses makes it a valuable diagnostic tool in many pathological conditions. In our study, SAA concentrations showed significant fluctuations that correlated with the titre intensity of *B. bronchiseptica* infection. This marked response provides evidence for an inflammatory reaction of the host against the bacterial infection studied. Our findings demonstrate that measuring SAA concentration is a specific test for *B. bronchiseptica* infection, even though acute phase proteins (APPs) can respond differently to various pathogens. However, given that the magnitude and duration of SAA elevation may vary depending on the pathogen and host response, more extensive studies are required to elucidate this relationship fully.

6 | Conclusion

In conclusion, *B. bronchiseptica* is seen as a zoonotic pathogen with heterogeneous clinical findings such as isolated bacteraemia and peritonitis as well as persistent cough and respiratory symptoms, and it is a pathogen that is becoming more and more known day by day. The causative agent of respiratory tract disease was detected in shelter dogs in Van province. Even the SAA concentrations of the causative agent were measured and analyzed (Independent 2-sample T-test), and it was concluded that it could be used as a valuable tool in both diagnosis and monitoring the disease's treatment process. It is predicted

that stray dogs may play an essential role in transmitting *B. bronchiseptica* to domestic dogs and may even infect humans. Therefore, to control zoonotic disease agents, vaccination against *B. bronchiseptica* and keeping stray animals under control can be considered among the measures to be taken by the authorities. In addition, it is essential to reveal the presence of the causative agent in the regions to combat the disease. This study is expected to shed light on possible studies that could be carried out in Turkey or Van province. In addition, more comprehensive studies are needed to determine the potential transmission of the disease agent.

Author Contributions

Kadir Akar: Motivation/concept, design, data collection and processing, analysis and interpretation, literature review, writing the article. **Gökçenur Sanioğlu Gölen**: Motivation/concept, analysis and interpretation, literature review, writing the article. **İsmail Hakkı Ekin**: Control/supervision, data collection and processing, critical review.

Ethics Statement

Ethical approval of the study was obtained from Van Yüzüncü Yıl University Animal Researches Local Ethics Committee (Decision no: 2023/13-10), Van, Turkey.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data available on request from the authors.

Peer Review

The peer review history for this article is available at https://publons.com/publon/10.1002/vms3.70323

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