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SARS-CoV-2 antibodies seroprevalence in dogs from France using ELISA and automated western blotting assays

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ABSTRACT

Dogs are occasionally receptive to SARS-CoV-2. They develop few or no clinical signs. Epidemiosurveillance of SARS-CoV-2 in dogs requires testing to distinguish it from other canine coronaviruses. Over the last year, significant progress has been made in the diagnosis of SARS-CoV-2, enabling its surveillance in humans and animals. Here, using ELISA and automated western blotting (AWB) assays, we performed a longitudinal study on 809 apparently healthy dogs from different regions of France to investigate anti-SARS-CoV-2 antibodies. There were three principal groups: (i) 356 dogs sampled once before the pandemic, (ii) 235 dogs sampled once during the pandemic, and (iii) 218 dogs, including 82 dogs sampled twice (before and during the pandemic), 125 dogs sampled twice during the pandemic and 11 dogs sampled three times (once before and twice during the pandemic). Using ELISA, the seroprevalence was significantly higher during the pandemic [4.9% (22/453)] than in the pre-

pandemic period [1.1% (5/448)]. At least 8 ELISA-seroconversions were observed among the 218 dogs sampled twice. ELISA positive sera before the pandemic were not confirmed in serial testing by AWB, which suggests a possible cross-reactivity of the ELISA, probably with other canine coronaviruses. No significant difference was observed between these two serological tests (Q=1.455, p=0.228). Positive correlation was observed between the SARS-CoV-2 seroprevalence in dogs and the incidence of the infection in humans. The AWB could be used as a second line assay to confirm the doubtful and discrepant ELISA results in dogs. Our findings confirm the previous experimental models concerning the receptivity of dogs to SARS-CoV-2. They suggest the weak or absence of the virus transmission from the infected to noninfected dogs or humans. However, the new variants with multiple mutations could adapt to dogs; this hypothesis cannot be ruled out in the absence of canine SARS-CoV-2 genomic data.

Keywords: COVID-19, SARS-CoV-2, Serology, Dog, Epidemiosurveillance, One Health

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1. Introduction

Severe acute respiratory syndrome, caused by SARS-CoV-2 coronavirus, a new emergent variant involved in epidemic disease first identified, in November 2019, in Wuhan city (Hubei province), China [1,2]. A few months later, the World Health Organization declared a worldwide pandemic disease. By the end of February 2021, more than 111 million cases and 2.46 million deaths were recorded worldwide [3]. In France, the first human cases were diagnosed in late January 2020. One year later (February 2021), the cumulative incidence for France reached almost 3.59 million, including 84,147 deaths [3,4].

Phylogenetically, SARS-CoV-2 is closely related to SARS CoV (or SARS-CoV-1), previously involved in the epidemic of 2003, and to the BatCoV, a Betacoronavirus found naturally in bats [5,6]. The scientific community believes that SARS-CoV-2 has a zoonotic origin from bats, while the intermediate host between bats and humans is not yet known [2,5,6]. Due to the presence of specific receptors for SARS-CoV-2 virus within the respiratory tract of mustelids (i.e. ferret and mink), these being the most receptive species under both experimental and natural conditions [7]. Globally, coronaviruses are widespread in animal fauna (i.e. birds, pigs, ruminants, dogs, cats, etc.) [2,5,6,7,8,9,10]. Since the 1970s, Alpha and Betacoronavirus have been highlighted respectively as agents for canine enteritic coronavirus (CECoV) and the respiratory coronavirus (CRCoV) [11,12]. However, dogs are occasionally receptive to SARS-CoV-2 with only 31 cases diagnosed worldwide by specific analyses (RT-qPCR) at the end of 2020 in Hong Kong, USA, Japan and Argentina [9,13]. Dogs infected with SARS-CoV-2 have few or no clinical symptoms [13]. The epidemiological surveillance of SARS-CoV-2 in dogs requires reliable serological methods to distinguish between the SARS-CoV-2 and other canine coronavirus. Advances in the diagnosis of SARS-CoV-2 have been made over the past year and surveillance of its circulation in humans and animals is now possible. Here, we performed a longitudinal study of the seroprevalence of SARS-CoV-2 in apparently healthy dogs from different regions of France in order to highlight their sentinel role during this pandemic.

2. Materials and methods

2.1. Dogs

A total of 809 dogs from France were included in this study (i.e. Bouches-du-Rhône, Marne, Lot, Var, Vaucluse, Corsica and French Guiana), from which 448 serum samples were sampled prior to the SARS-CoV-2 pandemic (from 2006 to January 2020) and 453 during the

pandemic (from February 2020 to February 2021). Of those, 559 (69%) consist of military working dogs (MWD), mainly male Belgian shepherds and German shepherds, aged from one to ten years, and 250 (31%) companion dogs (adults of both sexes, mostly living in shelters). Dogs were allocated into three groups: (i) 356 dogs were sampled once before the pandemic, (ii) 235 dogs were sampled once during the pandemic and (iii) 218 dogs, including 82 dogs sampled twice (before and during the pandemic), 125 dogs sampled twice during the pandemic and 11 dogs sampled three times (once before and twice during the pandemic). A total of 901 blood samples were collected using a 3.5 mL vacuum tube with serum separating gel. Canine sera were harvested and stored at - 20°C or + 4°C until analysis.

2.2. ELISA assay

All sera were subjected to the screening for antibodies against SARS-CoV-2 using ID Screen® SARS-CoV-2 Double Antigen Multi-species (Innovative Diagnostics, Grabels, France) following the manufacturer's instructions. The test consists of an enzyme-linked immunosorbent assay ELISA, targeting multispecies (i.e. minks, ferrets, cats, dogs, cattle, sheep, goats, horses and all other receptive species) antibodies directed against the major nucleocapsid protein of SARS-CoV-2. Plates were sensitized with a purified recombinant N antigen. Optical density (OD) was measured at 450 nm using Multiskan GO software (Thermo Scientific, Waltham, MA, USA). The test was validated when the optical density of positive control (OD_{PC}) was ≥ 0.35 and a mean ratio of positive (OD_{PC}) and negative (OD_{NC}) control is higher than three. The optical density of each sample (OD_N) was used to calculate the S/P ratio score (expressed as a %) where $S/P = 100 * (OD_N - OD_{NC})/ (OD_{PC} - OD_{NC})$. Samples tested by ELISA were considered positive when the S/P ratio score is higher than 60% and doubtful when the P/S percent ranges between 50 and 60%, while samples displaying an S/P score lower than 50% by ELISA were considered as negative.

2.3. SARS-CoV-2 antigen preparation and automated western blotting (AWB) assay

The strain SARS-CoV-2 IHUMI2 (lineage 20a) was used to produce SARS-CoV-2 antigens as previously described [14]. Briefly, virions were purified and harvested from the in vitro infected cells then fractionated with TS buffer (7 M Urea, 2 M Thiourea, 4% Chaps) to release SARS-CoV-2 antigens. The released antigens were concentrated with the Amicon 3 kDa filter (Merck KGaA, Darmstadt, Germany) before being used in western blotting assay [14,15].

The Jess^{TM} Simple Western automated nano-immunoassay system (ProteinSimple, San Jose, CA, USA, a Bio-Techne Brand), a capillary-based size separation of proteins [15] was used with an internal system control to evaluate the absolute serological response to viral antigens from all ELISA-positive samples. Canine sera were processed according to the manufacturer's standard method for 12-230-kDa Jess separation module (SM-W004). The Edouard's protocol [15] was adapted to detect canine antibodies directed to SARS-CoV-2. Briefly, a mixture of SARS-CoV-2 antigens, fluorescent molecular weight markers and 400 mM dithiothreitol (ProteinSimple) was prepared at final concentration of 0.25 µg/µL, then denatured at 95°C for 5 minutes. Viral protein migration was performed through the separation matrix at 375 volts for both SARS-CoV-2 antigens and Ladder (12-230-kDa PS-ST01EZ). Separated proteins were immobilized using photoactivated capture chemistry within the ProteinSimple proprietary [15]. Finally, canine sera diluted at 1:2 were incubated for 60 minutes followed by a wash step and a 30 minutes incubation within a multi-species HRP-conjugated anti-Fc fragment of IgG/IgM/IgA antibodies (Innovative Diagnostics, Grabels, France). The peroxide/luminol-S (ProteinSimple) was used for the chemiluminescent revelation. The Compass Simple Western software (version 5.0.1, ProteinSimple) was used for the automatic calculation of the heights (chemiluminescence intensity), area and signal/noise ratio as well as to capture the digital image of the capillary chemiluminescence.

2.4. Statistical analysis

Comparison between dog's populations was performed using Fisher's exact and Chi-squared tests. The Mc Nemar test was used to compare between ELISA and AWB assays. All statistical analysis was performed using Addinsoft software (XLSTAT 2018: Data Analysis and Statistical Solution for Microsoft Excel, Paris, France). A p-value < 0.05 was considered statistically significant.

3. Results

3.1. ELISA antibody detection

In total, of the 448 pre-pandemic sera collected, 4 (0.9%) were ELISA positive and 1 (0.2%) was inconclusive. This confirms a measured specificity of 99.1% [97.7 – 99.7] for the ELISA. While of the 453 sera collected during the pandemic, 20 (4.4%) were positive and 2 (0.5%) were considered doubtful. The infection rate was significantly higher during the pandemic compared to the period before the pandemic; this was observed for all sera samples (Table 1). Furthermore, at least 8 ELISA-seroconversions among the 218 dogs during the pandemic were observed (Table 2). During the pandemic, a total of 17 (4.3%) out of 397 MWD and 6 (10.7%) out of 56 companion dogs were reacted within ELISA test, which corresponds to a significant difference (Khi2=4.213 - p=0.04) between these two populations. Fourteen (11.1%) out of 126 dogs sampled in February 2021 from the South-East area scored positive. A lower prevalence of 3.1% (3/95) was recorded in the South-West compared to that recorded in the South-East (Khi2=4.7 - $p \leq 0.05$) (Table 3).

3.2. Automated western blot results

Among the 41 serum samples listed in table 2, 31 of them were assessed by the AWB, including 27 ELISA-positive sera, one doubtful serum and 3 ELISA-negative sera. In addition, three other ELISA-negative sera were also tested. AWB yielded the detection of 17 (63%) out of the 27 ELISA-positive sera (including doubtful sera). In addition, 3 ELISA-negative sera were found positive within AWB. One of them was a MWD (D14) which exhibited an S/P ratio of 49%, and the two other sera collected at one week apart (D26). Globally, all AWB-positive sera were sampled between the period ranging from January 2020 to February 2021. While no ELISA-positive sera collected before the pandemic or negative controls were detected by the AWB (Figure 1). No significant difference was observed between these two assays (Q=1.455, p=0.228). Finally, all AWB-positive sera yielded a prominent 56-kDa band interpreted as the nucleocapsid, while no bands were detected for the other major dominant proteins, such as the protein S (i.e. 170 kDa), S1 (i.e. 110 kDa) and S2 (i.e. 90 kDa) (Figure 1).

4. Discussion

To date, studies investigating SARS-CoV-2 in dogs are scarce, probably due to the lower susceptibility of dogs to this infection and the focus of research on the human disease. In France, only two serological studies have been carried out on dogs. One study involved 12 dogs of SARS-CoV-2-positive owners. In this study, no positive dog was detected using the luciferase immuno-precipitation assay [16]. The second study was carried out using the microsphere immunoassay. Authors reported 2 (15.4%) seropositive dogs among 13 of SARS-CoV-2-positive owners, while no positive-dog was found within 22 other dogs of owners with an unknowing SARS-CoV-2 status [17]. In Italy, the antibody neutralization assay was used for the surveillance of SARS-CoV-2 infection in 451 dogs during the pandemic, and 15 (3.3%) dogs were found seropositive [18]. In Wuhan city (China), 16 (1.7%) positive dogs were

detected among the 946 tested during the pandemic using a newly developed double-antigen sandwich ELISA assay [19]. In Croatia, a survey reported that 7.6% of dogs (13/172) were positive by ELISA test [20]. In Texas, USA, 15.3% of 59 dogs were positive for SARS-CoV-2 by RT-PCR and genome sequencing or neutralizing antibodies, in homes where at least one human case of COVID-19 was diagnosed [21]. In Spain, canine seroprevalence (ELISA) was overall 16.7% (10/60) but it was higher (25% - 5/60) in dogs living in COVID-19-positive households, indicating their susceptibility to SARS-CoV-2 infection [22]. These discrepancies in results between the different studies may be related to the sensitivity of the different assays. The results of this comprehensive study of SARS-CoV-2 infection in companion and military working dogs sampled before and during the pandemic in areas of active human viral transmission made it possible to evaluate the specificity of the ELISA and AWB tests. The same ELISA test used in our study detected anti-SARS-CoV-2 antibodies in the serum of a cat with PCR positive, living in a household in Chile, where a human was infected [23].

In our study, the ELISA we used detected 1.1% of 448 pre-pandemic sera. This highlights the possible cross reactivity with other canine coronaviruses, probably the Betacoronavirus of dogs [24]. On the other hand, the seroconversion of 8, as well as the significant increase in seroprevalence in dogs during the pandemic (i.e., 4.9% out of 453 dogs tested), particularly in the Bouches-du-Rhône region, a high endemic area for human SARS-CoV-2 infection (www.cascoronavirus.fr), could explain the occurrence of SARS-CoV-2 infection in dogs. On the other hand, the AWB assay yielded the detection of 62.7% ELISA-positive sera. However, all of them were sampled between the periods ranging from January 2020 to February 2021, which is in line with the outbreak of the pandemic in France. In addition, some inconsistencies were also observed between these two assays. For example, some dogs with high ELISA S/P ratio sampled before the pandemic (i.e. dog D1 and D2) or even during the pandemic (i.e. dog D6 and D7) gave a negative AWB result, whereas some ELISA-negative or doubtful sera with

low ELISA S/P ratio (i.e. dog D14, D22, D26 and D29) were positive using AWB assay (Fig.1). Though few canine sera were herein tested by the AWB, which may represent a limitation of the assay, all AWB-positive sera were sampled during the pandemic which suggests the specific detection of antibodies to SARS-CoV-2 in dogs. The discrepancy between these two assays could be explained by the type of antigens used for each assay. ELISA test was developed on the basis of a truncated N recombinant antigen from the viral nucleocapsid which probably provided the detection of conformational epitopes that could also be shared with the other coronaviruses. In contrast, the AWB was based on the integral SARS-CoV-2 nucleocapsid antigens which may react only with the linear epitopes [25]. However, the clear-cut decision regarding the specificity of the AWB assay cannot be ruled out in the absence of a reliable gold standard, since the possible cross-reaction has already been described with other human Betacoronavirus within the AWB assay [26].

The AWB assay based on the purified virus antigens was first adapted for the diagnosis and the evaluation of the human immune-response against SARS-CoV-2 antigens. The assay proved to be effective principally in detecting antibodies to nucleocapsid proteins [15]. Our results showed that the AWB yielded only the detection of antibodies against the nucleocapsid proteins from all positive dogs. However, we do not know whether this is related to the lower sensitivity of AWB to spike virus proteins in dogs.

Despite the receptivity of dogs to SARS-CoV-2 infection under experimental conditions [26], they were unable to transmit the virus [7,9,10]. Our results indicated that, in spite of the presence of positive dogs in kennels, there were most probably few infected animals. Thereby, this suggests that dogs do not transmit the virus, which may be due to the poor viral replication in dogs [26]. On the other hand, previous studies have demonstrated the presence of a few differences between human and canine angiotensin-converting enzyme 2 (ACE2), the interactive receptor within the spike protein of the SARS-CoV-2 [9]. However, recent studies

have demonstrated the continuous emergence of new SARS-CoV-2 with multiple spike protein mutations. It is not known whether dogs infected with these new variants could transmit the virus to other animals or to humans [27,28,29]. In March 2021, a study carried out on British dogs reported for the first time canine and feline infections with the SARS-CoV-2 B.1.1.7 variant in addition to some of these pets suffering from myocarditis [30].

5. Conclusion

The AWB assay, previously standardized as first or second line method to confirm the diagnosis of SARS-CoV-2 from human patients, could also be used as a second line assay to confirm negative, doubtful and discrepant ELISA results in dogs. These findings along with the results from the previous experimental models of SARS-CoV-2 in dogs confirm the receptivity of dogs to SARS-CoV-2 infection. They also suggest the absence of the virus transmission from infected to non-infected dogs as well as to humans. In the absence of genomic data on SARS-CoV-2 in dogs, the hypothesis that new SARS-CoV-2 variants with multiple mutations in the spike protein could induce adaptation of the virus to dogs cannot be ruled out.

Authors' contributions

BD and YL conceived the original paper. BD, YL and HM wrote the initial draft. BD, YL, PS, SWG, JG, VA, CL and JLM collected the blood samples. BD, YL, PS, HM and YS carried out analyzes in the laboratory. BD, YL, HM, SWG, LC, PP, JLM and DR extensively revised and approved the final version of the manuscript.

Ethics approval and consent to participate

All applicable international, national and military guidelines for the care and use of dogs were followed. The owners of the dogs have given their consent for the samples to be taken.

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Declaration of competing interest

There are no competing interests. Two authors (Loïc Comtet and Philippe Pourquier) are currently employees of Innovative Diagnostics company, however there were no conflicting interests that may have biased the work reported in this paper.

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Table 1

Seroprevalence of SARS-CoV-2 antibodies, detected with a double antigen ELISA test, in dogs from France before and during the COVID-19 pandemic (N=901).

	Samples of dogs	No. of positive (%)	No. of doubtful (%)	No. of sera reacted with ELISA (%)
Total sera	Before the pandemic (N=448)	4 (0.9%)	1 (0.2%)	5 (1.1%)
	During the pandemic (N=453)	20 (4.4%)	2 (0.5%)	22 (4.9%)
	Statistics	Khi2=10.84; p=0.001		
Sera sampled one time	Before the pandemic (N=356)	3 (0.8%)	0 (0%)	3 (0.8%)
	During the pandemic (N=235)	13 (5.5%)	1 (0%)	13 (5.5%)
	Statistics	Khi2=13.225; p<0.0001		
Dogs' activity	MWD	14 (3.5%)	3 (0.8%)	17 (4.3%)
	SD and PD	6 (10.7%)	0 (0%)	6 (10.7%)
	Statistics	Khi2=4.213; p=0.04		

SD: shelter dogs, MWD: military working dogs, PD: pet dogs

Table 2

Individual positive results of serological detection of SARS-CoV-2 infection by the double antigen ELISA test (N=28 dogs).

	Serum Id.	Dog Id.	Dog category	Location Department	Date of sample	ELISA DO	(S/P) %	ELISA result	WB result
Dogs collected once before the pandemic (N=356)	D699	D1	SD	French Guiana	01.2016	0.558	231,1	Positive	Negative
	D681	D2	SD	Corsica	03.2018	1.012	424,3	Positive	Negative
	D662	D3	MWD	Bouches-du-Rhône	10.2018	0.214	84,7	Positive	Not tested
Dogs collected once during the pandemic (N=235)	D95	D4	MWD	Marne	06.2020	3.89	278,1	Positive	Positive
	D132	D5	MWD		09.2020	0.901	61,2	Positive	Negative
	D306	D6	SD	Bouches-du-Rhône	02.2021	0.78	166,8	Positive	Negative
	D307	D7	SD		02.2021	0.764	162,9	Positive	Negative
	D312	D8	SD		02.2021	0.62	127,8	Positive	Positive
	D697	D9	SD		02.2021	0.197	77,4	Positive	Positive
	D357	D10	SD		02.2021	0.739	156,8	Positive	Positive
	D358	D11	SD		02.2021	0.52	103,4	Positive	Positive
	D400	D12	MWD		02.2021	1.648	435,2	Positive	Positive
	D441	D13	MWD		02.2021	0.82	207,7	Positive	Positive
	D568	D14	MWD		02.2021	0.332	49,0	Negative	Positive
	D627	D15	MWD	Lot	02.2021	0.41	61,2	Positive	Negative
	D646	D16	MWD		02.2021	0.421	62,9	Positive	Positive
	D438	D17	MWD	Bouches-du-Rhône	02.2021	0.29	62,1	Positive	Negative
Dogs collected twice, once before and once during the pandemic (82)	D560	D18	MWD	Bouches-du-Rhône	06.2018	0.043	0,7	Negative	Not tested
	CR10		MWD		10.2020	0.344	55,3	Doubtful	Not tested
	D211	D19	MWD		11.2019	0.065	1,4	Negative	Not tested
	D210		MWD	Marne	05.2020	1.166	96,0	Positive	Negative
	D23	D20	MWD		01.2020	1.31	83,0	Positive	Positive
	D24		MWD		06.2020	1.373	87,2	Positive	Positive
Dogs collected twice during the pandemic (N=125)	D229	D21	MWD	Marne	03.2020	0.21	13,8	Negative	Negative
	D228		MWD		07.2020	0.821	66,4	Positive	Positive
	D57	D22	MWD		04.2020	0.435	25,0	Negative	Not tested
	D58		MWD		09.2020	0.89	55,2	Doubtful	Positive
	D96	D23	MWD		06.2020	0.292	17,0	Negative	Not tested
	D97		MWD		10.2020	0.704	46,9	Negative	Not tested
	D175	D24	MWD		07.2020	0.069	0,8	Negative	Negative
	D696		MWD		01.2021	0.106	38,7	Negative	Negative
	LD82	D25	MWD	Bouches-du-Rhône	10.2020	0.314	27,1	Negative	Not tested
	D417		MWD		02.2021	0.78	196,7	Positive	Positive
	D660	D26	PD		11.2020	0.049	14,5	Negative	Positive
	D661		PD		12.2020	0.105	38,3	Negative	Positive
Dogs collected three times, once before and twice during the pandemic (N=11)	LD84	D28	MWD	Bouches-du-Rhône	06.2018	0.101	3,7	Negative	Not tested
	LD83		MWD		10.2020	1.503	157,6	Positive	Positive
	D408		MWD		02.2021	0.512	123,1	Positive	Positive
	LD86		MWD		06.2018	0.111	4,8	Negative	Not tested
	LD85	D29	MWD		10.2020	0.592	57,6	Doubtful	Positive
	D382		MWD		02.2021	0.549	133,2	Positive	Positive
Negative controls	D569	D30	MWD	Lot	02.2021			Negative	Negative
	D570	D31	MWD		02.2021			Negative	Negative
	D571	D32	MWD		02.2021			Negative	Negative

SD: shelter dog, MWD: military working dog, PD: pet dog

Table 3

Comparison of seroprevalences (ELISA) of SARS-CoV-2 infection in dogs from the French departments of Bouches-du-Rhône (South-East) and Lot (South-West) in February 2021, and the correlation with the COVID-19 incidence in humans.

Location Department	Number of dogs	Number of ELISA negative dogs	Number of ELISA positive dogs	Canine seroprevalence (%)	Human COVID-19 incidence rate per 100,000 inhabitants as of 02/16/2021	Seropositivity rate of COVID-19 virological tests carried out in humans (%) as of 02/16/2021
Bouches-du-Rhône (South-East)	126	113	14	11.1	332	6.6
Lot (South-West)	95	92	3	3.1	100	2.9
Total	221	205	17	7.7		
Statistical comparisons				Chi-square = 4.7 between these two groups - significant difference $p \leq 0,05$	Chi-square = 124 between these two groups - very significant difference	

Fig. 1. Results of the automated western blotting assay of SARS-CoV-2 infection in dogs from France, before and during the COVID-19 pandemic (N=32).