



Research article



Sensitivity and specificity of *in vivo* COVID-19 screening by detection dogs: Results of the C19-Screendog multicenter study

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ABSTRACT

Trained dogs can recognize the volatile organic compounds contained in biological samples of patients with COVID-19 infection. We assessed the sensitivity and specificity of *in vivo* SARS-CoV-2 screening by trained dogs.

We recruited five dog-handler dyads. In the operant conditioning phase, the dogs were taught to distinguish between positive and negative sweat samples collected from volunteers' underarms in polymeric tubes. The conditioning was validated by tests involving 16 positive and 48 negative samples held or worn in such a way that the samples were invisible to the dog and handler. In the *screening* phase the dogs were led by their handlers to a drive-through facility for *in vivo* screening of volunteers who had just received a nasopharyngeal swab from nursing staff. Each volunteer who had already swabbed was subsequently tested by two dogs, whose responses were recorded as positive, negative, or inconclusive. The dogs' behavior was constantly monitored for attentiveness and wellbeing.

All the dogs passed the conditioning phase, their responses showing a sensitivity of 83–100% and a specificity of 94–100%. The *in vivo* screening phase involved 1251 subjects, of whom 205 had a COVID-19 positive swab and two dogs per each subject to be screened. Screening sensitivity and specificity were respectively 91.6–97.6% and 96.3–100% when only one dog was involved, whereas combined screening by two dogs provided a higher sensitivity. Dog wellbeing was also analyzed: monitoring of stress and fatigue suggested that the screening activity did not adversely impact the dogs' wellbeing. This work, by screening a large number of subjects, strengthens recent findings that trained dogs can discriminate between COVID-19 infected and healthy human subjects and introduces two novel research aspects: i) assessment of signs of fatigue and stress in dogs during training and testing, and ii) combining screening by two dogs to improve detection sensitivity and specificity.

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Using some precautions to reduce the risk of infection and spillover, *in vivo* COVID-19 screening by a dog-handler dyad can be suitable to quickly screen large numbers of people: it is rapid, non-invasive and economical, since it does not involve actual sampling, lab resources or waste management, and is suitable to screen large numbers of people.

1. Introduction

Since 2020, the emergence and fast spread of the SARS-CoV-2 virus has taken a devastating toll, with 6.3 million deaths and more than 534 million confirmed cases of COVID-19 infection in the world [1]. Currently, the highly infective omicron variant is rampant even in countries where most individuals have received two or three vaccine doses or have been infected with earlier variants [2]. Improving interventions such as contact tracing, testing, diagnosis, and isolation is therefore still crucial to avert virus spread.

In the past few decades, volatile organic compounds (VOCs) have been attracting considerable interest as potential biomarkers of human disease [3]. Their production has been investigated in cancer [4–9] as well as metabolic [10–12] and infectious diseases [3, 13–16].

The sense of smell of dogs is very highly developed, due to the combination of a large number of genes coding for olfactory receptors (ORs) (1100 vs 350 in humans), a large olfactory epithelium (18–150 cm² vs 3–4 cm²), a high number of ORs (50–300 million vs 5–6 million) and a large olfactory bulb (3 cm vs 1 cm) [16]. Hitherto, it was assumed that in humans >50% of olfactory receptor genes are pseudogenes, whereas Menashe et al. [17] using probabilistic Classifier for Olfactory Receptor Pseudogenes predicted that even ~70% of human OR may be non-functional pseudogenes. The canine OR subgenome is estimated to have 12% pseudogenes, which is considerably less than for humans [18], thus the percentage of functional OR genes in canines is larger. VOC concentrations are measured as parts per billion/trillion (ppb/ppt) in human breath and as parts per million (ppm)/ppb in human blood and urine [19]. Notably, most VOCs are found in the volatilome at concentrations that are well in the range of a dog's detection ability of 1 ppt. Such sensitivity is three orders of magnitude lower than that of current instruments [20], *i.e.* gas and liquid chromatography (GC, LC, HPCL) coupled with mass spectroscopy [21], which are also quite expensive.

In the past two years, a number of research groups have documented the ability of dogs to detect the SARS-CoV-2 infection by smelling samples of saliva, tracheobronchial secretions or axillary sweat or single-use disposable masks [22]. The first randomized, double-blind controlled study, which involved saliva or tracheobronchial secretions and 8 detection dogs, reported a sensitivity of 82.63% and a specificity of 96.35% [23]. In contrast, a study of around 2000 saliva and sweat samples failed to meet the World Health Organization diagnostic accuracy requirements for COVID-19 rapid antigen tests of ≥80% sensitivity and ≥97% specificity [24]. In a multicenter study, 18 dogs that had been trained to recognize the smell of axillary sweat collected from hospitalized subjects with COVID-19 and other diseases (198 samples), achieved a success rate of 83–100% [25]. In another study of 241 subjects (109 positive, 132 negative) sensitivity was 89.6% and specificity 83.9% [26]. In November 2020, in an editorial in *Nature*, Holly Else stated that the validation and publication of these data and protocols could be useful to establish screening by sniffer dogs as an alternative method to identify subjects with COVID-19 infection in public or crowded places or in poor countries [27]. Two further studies with dogs, published respectively in September 2021 and March 2022, demonstrated that *in vivo* screening of individuals [28] or of skin swabs [29] achieved accurate results also in real-life situations and in crowded public places.

Dogs can be trained for a wide range of purposes including search and rescue operations, animal-assisted interventions (AAIs) and military operations [30], where they excel without experiencing high levels of anxiety or stress [31–33]. However, there is scant information to determine whether training for and performing COVID-19 screening is stressful for dogs. During AAI, it has been demonstrated that dogs show many behaviours belonging to the normal repertoire of the species. Even if the activity of COVID-19 screening can be compared to that of AAI projects, there is scant information to determine whether training for and performing COVID-19 screening is stressful for dogs. Understanding the emotional state of animals and highlighting any signal of stress is crucial to maintain the wellness of the animals and to enhance the probability of successful testing.

This study was denominated C19-Screendog project. Its aims are i) to validate a safe protocol for the operant conditioning and training of sniffer dogs and ii) to establish whether such dogs can achieve rapid *in vivo* screening of subjects with SARS-CoV-2 infection in real-life situations with high sensitivity and specificity and iii) to appraise dog wellbeing assessing signs of fatigue and stress during training and *in vivo* screening.

2. Materials and methods

2.1. Study participants

From May to December 2021, a total of 1415 volunteers were enrolled by ASUR Marche AV3 and ATS Sardegna, Sassari (Italy) to participate in the C19-Screendog project, which was approved by the local ethics committees (Regione Marche: CERM, no. 2021/219; ATS Sardegna: no. 344/2021/CE). In particular, i) 164 subjects aged 18–77 years, 41 positive and 123 negative for SARS-CoV-2, were involved in dog operant conditioning and validation, providing sweat samples or volunteering to be screened; and ii) 1251 individuals aged 18–99 years, 205 positive and 1046 negative for SARS-CoV-2, were involved in *in vivo* screening at a drive-through testing center. Participants were enrolled by contact tracing health offices among those requiring a nasopharyngeal swab for suspicious symptomatology or for coming into contact with COVID-19 infected subjects and were booked for a test at a convenient drive-through facility.

All subjects provided their informed consent to participate. The only exclusion criterion was age <18 years. The anamnestic and clinical data of all participants were recorded in a medical report form ([Supplementary material 1](#)).

2.2. Sweat sampling

Sweat was collected by the donors themselves by holding a Getxent tube (3.5 cm long; Getxent, Switzerland) to their armpit for 10 min; they then placed the tube into a bar-coded polypropylene test tube which they handed to the operator. Getxent tubes are specially designed to absorb VOCs, they are odorless, do not contain latex or other allergenic substances and are widely used in scent dog research. The sweat samples were then linked to the test result of each subject's nasopharyngeal swab. Samples, after being collected, were anonymized with alphanumeric codes, stored for two days at a temperature of 4 °C in the laboratory before sending to the dog handlers in a refrigerated atmosphere. In turn, dog handlers stored samples at a temperature of 4 °C for no more than 3 months, allowing the samples to equilibrate at room temperature for 30 min before the training session and placing again them at 4 °C thereafter. Otherwise, samples were stored in the laboratory at -80 °C for longer-term preservation.

In all phases involving the dogs, the Getxent tubes were placed into small metal boxes and handled with sterile gloves and tweezers. The boxes had two interchangeable lids: a perforated lid for use during conditioning and training that allowed VOC escape and avoided direct contact of the tube with both human skin and dog nose, and a solid one for storage.

2.3. Characteristics of the dog-handler dyads

The handlers recruited for this study are experienced trainers of AAI dogs. All dyads (dog and handler) belong to or are affiliated with Progetto Serena a.p.s., a non-profit organization involved in sniffer dog training for diabetes screening (www.progettoserenaonlus.it), and are managed directly by its founder, Dr. Roberto Zampieri, who developed the operant conditioning protocol based on previous research [23,25].

2.4. Dog selection

We asked experienced dog trainers who were affiliated to Progetto Serena a.p.s., to select five well-behaved dogs with an easy character. The dogs were selected according to AAI ministerial guidelines (https://www.salute.gov.it/portale/documentazione/p6_2_5_1.jsp?lingua=italiano&id=276). Briefly, after a health, skill, and aptitude assessment, five sociable, docile, curious, and collaborative dogs were selected for the study. Prior to enrolment, the handlers were asked to complete the Canine Behavioral Assessment & Research Questionnaire (C-BARQ, <https://vetapps.vet.upenn.edu/cbarq/index.cfm>), a standardized evaluation tool that assesses canine personality and highlights behavioral problems [34,35]. The dogs were trained to respond to basic commands of their handler (e.g., “sniff” and “come”), to disregard distractions, and to be unaffected by a variety of human behaviors that may occur in medical settings, like an anxious person speaking in a high tone of voice.

The dogs recruited for the study were five intact females (two Labrador retrievers, a Corsican, a Maltese, and a mixed breed) aged 1.5–12 years. According to the C-BARQ, all were easy to train, and none showed stranger, owner, or dog directed aggression, chasing behavior or separation anxiety (Table 1).

2.5. Dog training and in vivo screening

In the first phase, operant conditioning and dog validation, the dogs were trained to distinguish between axillary sweat samples collected from subjects with and without COVID-19 infection.

In the subsequent phase, *in vivo screening*, a cohort of 1251 volunteers, who had just received a nasopharyngeal swab at a drive-

Table 1
Sex, breed, age, and personality (as assessed by the C-BARQ) of the five detection dogs used in the study.

Name	Sex	Breed	Age (years)	Personality
Cloe	Female	Labrador retriever	1.5	Low level of aggressiveness toward dogs, no stranger-directed, owner-directed or dog-directed aggression, very easily trainable and with low levels of chasing and stranger-directed fear, no separation anxiety, no touch sensitivity, little levels of excitability and energy.
Dayanne	Female	Corso	1	Easily trainable, not very aggressive towards dogs and strangers, not aggressive towards the owner, not inclined to predatory behavior, not afraid of strangers, no separation anxiety, she does not often seek the attention of humans, very excitable, moderate concerning about barking and snapping at flies.
Shaila	Female	Mixed breed	9	Easily trainable, not aggressive towards dogs, strangers, and the owner, not inclined to predatory behavior, not afraid of strangers, no separation anxiety, she does not often seek the attention of humans, not very excitable.
Wave	Female	Labrador retriever	1	Easily trainable, not aggressive towards dogs, strangers, and the owner, not inclined to predatory behavior, not afraid of strangers, no separation anxiety, she does not often seek the attention of humans, not very excitable.
Nenna	female	Maltese	12	Easily trainable, not aggressive towards dogs, strangers, and the owner, not inclined to predatory behavior, not afraid of strangers, no separation anxiety, she does not often seek the attention of humans, not very excitable.

through facility, were screened by two dogs in succession.

2.6. Phase 1: Operant conditioning and validation of conditioning

Operant conditioning. The operant conditioning and training protocol was based on the sniff-reward method. All the dogs recruited for this study had already been previously trained to recognize the “sit” command with a slight upward movement of the handler’s hand. As a general rule, in case of a new dog recruitment, this instruction is essential before proceeding with the conditioning protocol. The conditioning protocol involved 3 steps and took about 2 months of daily dyad exercises (20 min per day).

Step 1. Positive and negative samples were proposed to the dogs by their handlers separately one by one with the command “search” as follows: identification of a positive sample, then identification of a negative sample followed immediately by the identification of a positive sample. When a positive sample was proposed, the dog smelled it and then looked at his handler who, with the movement of the hand, invited him to sit and rewarded with a treat; when a negative sample was proposed, the movement of the hand was not carried out and the dog was moved using a praise. In this conditioning phase each dog was presented with 5 different positive negative samples for a total of 10 samples until only the positive samples were indicated by the dogs with a “sit” therefore, without error (100% of correct responses). The reward was given only for the correct indication of positive samples. This step is variable in time depending on the dog, but generally needs no more than 10 days of repeated exercises for at least 20 min a day.

Step 2. After the dog had achieved 100% of correct responses, it was presented with a rotating sample holder, where it was required to identify the positive sample and ignore 3 negative samples. This phase used repeatedly 5 sets of samples, different from those used in step 1 (each composed of one positive and 3 negative) and all from different subjects (in total, 5 positive and 15 negative), until the dog achieved 100% of correct identifications of all samples in a single session with the rotating sample holder.

Step 3. The next step was *in vivo* sample screening (samples held by SARS-CoV-2 negative tested volunteers). We hypothesized that the use of volunteers (helpers) better educates the dog to the VOCs of positive individuals (samples) among a multitude of other VOCs (e.g., of hormones, soaps, family members). Here, the task involved initially detecting a positive sample held in one hand (right or left hand randomly) by a helper volunteer standing in front of the dyad. The dog on leash slowly circled once and sniffed the person and indicated sample location, without physically touching the screened person. The task was repeated more times until the dog performed it without errors or hesitations. In the next task, the helper also held a negative sample in the other hand. The dog was required to indicate only the positive one. After this task had been mastered (100% correct responses), the next task involved circling a group of subjects who were holding a positive or a negative sample, indicating only the positive sample. Samples used in this step were the same of Step 2.

2.7. Validation of the operant conditioning phase

To validate the conditioning phase, each dog was presented with 8 volunteers at once per session for a total of 8 different sessions. Each volunteer held out of sight either a positive or a negative (randomly placed in different places but from waist down, i.e. in pockets, under clothes) for a total of 2 positive and 6 negative samples per session (ratio 1:3; overall, 16 different positive and 48 negative samples). This phase was triple blinded, since neither the observers recording the alerts nor the handlers or the volunteers were aware of sample identity.

The results were analyzed to evaluate screening sensitivity and specificity (see Statistical analysis).

Sample recognition with a sensitivity and specificity >90% validated the conditioning phase.

2.8. Phase 2: *In vivo* screening

Once validated for operant conditioning, the dogs were led by their handlers to a drive-through facility, where they screened in succession a number of individuals (not their sweat samples) who had just received a nasopharyngeal swab for SARS-CoV-2 diagnosis taking care that the dog’s nose did not physically touch the subject to avoid risk of infection. To optimize VOC detection and avoid the distraction provided by grass, soil or bins in the vicinity, a gazebo was specially erected and paved with fake lawn. After their demographic data were recorded, the volunteers were interviewed to collect the necessary demographic and medical data, then they were asked to stand under the gazebo to be screened by the dogs.

Each dog was led to the subject on a leash (see Supplementary Video). Its indication was recorded as positive, negative, or dubious (that is when the dog gives an unclear signal or hesitates to “sit”) on the individual’s medical report form. The response was checked against the swab report, which became available 1–2 days later. We also evaluated the usefulness of having at least two dogs during the screening sessions because in case of one dubious signal, the reaction of a second dog may be resolute. Furthermore, in this way the dogs can alternate in the work and the fatigue can be reduced or prevented.

We calculated screening sensitivity and specificity for each dog and dog pair (see Statistical analysis).

The volunteers were screened in succession. The dogs were rested for 5 min every 5 subjects and then for 15 min every hour. Altogether, each dog screened up to 100 subjects in a day.

2.9. Dog behavior and wellbeing

Behavioral data were recorded and collected with the aim to appraise the wellbeing of dogs during such activity and highlight any possible early signal of stress. All sessions were recorded with camcorders to avoid observer interference. The dogs’ behavior in the videos

was monitored throughout the study, from the conditioning to the screening phase, by a trained veterinary researcher, who compiled an ethogram for each dog. The ethogram, developed by Corsetti and coworkers [36], assesses wellbeing and stress levels in dogs involved in AAI based on a set of 48 behavioral patterns, grouped into 11 behavioral categories, which include displacement activities, attention, olfactory investigation, vocal communicability, stereotyped behaviors, dominance, aggressiveness, submissive behaviors, affiliation, resting, and playfulness. Data were collected using the focal animal sampling method and 'all occurrences' recording [37] and subjected to a double examination by two trained veterinary researchers to highlight any possible sign of stress or fatigue.

All the procedures described in which dogs were involved falls out of the field of application of Legislative Decree no. 26 of 4 March 2014 (Implementation of the Directive 2010/63/UE on Protection of animals used for scientific purposes). In fact, following article 26 (paragraph 2, letter f) of the decree, this does not apply to practices not likely to cause pain, suffering, distress or prolonged damage equivalent to or greater than that caused by the insertion of a needle according to good practice veterinary. Since the dogs were used only for olfactory screening and no experimental procedures were carried out on these dogs, the authorization is not needed.

2.10. Statistical analysis

Categorical variables, including dog indications, were presented as frequencies and percentage and compared using Fisher's exact test or the chi-square test as appropriate. Continuous variables, i.e. participant age and time from first vaccination, were presented as median (interquartile range) and compared using Mann-Whitney's *U* test. Sensitivity was calculated as $[(\text{True Positives, TP})/(\text{TP} + \text{False negatives, FN})] \times 100$; specificity as $[(\text{True negatives, TN})/(\text{TN} + \text{False positives, FP})] \times 100$; correct classification rate as $[(\text{TN} + \text{TP})/(\text{TN} + \text{TP} + \text{FN} + \text{FP})] \times 100$. The 95% confidence intervals (CIs) for sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated using Wilson's method [38]. Interrater agreement was evaluated using Cohen's kappa [39]. The threshold of statistical significance was $p < 0.05$. All analyses were performed using R, version 4.1.2.

To estimate the required number of samples, a sensitivity threshold of COVID-19 detection of 90% was adopted, in line with the European Council Recommendation on a common framework for the use and validation of rapid antigen tests (<https://data.consilium.europa.eu/doc/document/ST-5451-2021-INIT/en/pdf>), which sets minimum performance requirements of $\geq 90\%$ sensitivity and $\geq 97\%$ specificity. Assuming alpha and power (1-beta) to be 0.05 and 90%, respectively, and a COVID-19 prevalence of 7% (as of April 2021), we calculated that 929 subjects would be required to achieve the desired sensitivity with a 15% margin of error.

For the study of dog behavior, the differences in behavioral patterns in the two phases were analyzed using the non-parametric Friedman test, which is applied to detect differences in outcomes across multiple test attempts. These analyses were performed with the SPSS software system.

3. Results

3.1. Validation of operant conditioning

After completion of the conditioning phase, the five dogs were subjected to validation as described in methods. In this validation step, when a dubious indication was provided by the dog a second attempt to correctly identify the same sample was performed immediately thereafter in the same session. If the problem persisted, the indication was considered as a positive response for the determination of test sensitivity and specificity and as an incorrect response for the calculation of the proportion of correctly classified samples. Analysis of the results of operant conditioning validation (Table 2) yielded a sensitivity and specificity $>90\%$ for 4 dogs. The fifth, Shaila, gave incorrect indications for 2 positive samples (in two different sessions) and 5 negative samples (in 4 different sessions); however, 2 of the individuals carrying negative samples had received the first dose of COVID-19 vaccination the day before the validation session, whereas another had a positive swab 2 days after his participation in the study. Replacement of these subjects resulted in correct reclassification of the 5 negative samples by Shaila, raising specificity to 94.4%. Thus, four out of five dogs passed the validation phase. However, also the fifth dog (Shaila) was admitted to the screening phase, based on the high specificity, the overall good sensitivity, and on the reduced number of positive samples that would not have allowed to achieve a precise estimation of the sensitivity in the validation phase.

4. Results of *in vivo* COVID-19 screening by sniffer dogs

Of the 1251 subjects who received a nasopharyngeal swab at the drive-through center from July to December 2021, 205 (16.39%)

Table 2

Sample screening performance of the five dogs in the validation of the operant conditioning phase.

	Cloe	Dayanne	Wave	Shaila	Nenna
No. of samples tested	48	52	40	48	48
No. of positive samples	12	14	10	12	12
Sensitivity (%)	100.0 (75.8, 100.0)	92.9 (68.5, 98.7)	100.0 (72.2, 100.0)	83.3 (55.2, 95.3)	91.7 (64.6, 98.5)
Specificity (%)	94.4 (81.9, 98.5)	94.7 (82.7, 98.5)	100.0 (88.6, 100.0)	94.4 (81.9, 98.5)	94.4 (81.9, 98.5)
Correct classification (%)	95.8	90.4	97.9	85.4	93.8

95% CI intervals reported in brackets.

Table 3
Demographic and anamnestic information of the subjects who received a nasopharyngeal swab at the drive-through center.

Variable	Total	Negative for COVID-19	Positive for COVID-19	p-value
N (%)	1251 (100%)	1046 (83.6%)	205 (16.4%)	–
Gender (males, %)		437 (41.8%)	114 (55.6%)	<0.001
Age (years)		49 (34–69)	39 (27–60)	<0.001
COVID-19 relatable symptoms (n, %)		437 (41.8%)	137 (66.8%)	<0.001
Close contacts with COVID-19-positive individuals (n, %)		446 (42.6%)	131 (63.9%)	<0.001
Comorbidities (n, %)		95 (9.1%)	14 (6.8%)	0.296
Cardiovascular disease		49	7	
Dementia		14	2	
Cancer (current or past)		6	1	
Respiratory disease		7	3	
Renal disease		6	2	
Autoimmune disorders		5	–	
Diabetes type 2		18	3	
Gastrointestinal disorders		4	–	
Other		2	–	
Vaccination status				<0.001
Not vaccinated (n, %)		303 (29.0%)	103 (50.2%)	
One dose (n, %)		47 (4.5%)	20 (9.8%)	
Pfizer-BioNTech		20	15	
AstraZeneca		3	–	
Moderna		23	5	
Janssen		1	–	
Two doses (n, %)		696 (66.5%)	82 (40.0%)	
Pfizer-BioNTech		548	53	
AstraZeneca		85	28	
Moderna		42	1	
Heterologous AstraZeneca/ Pfizer-BioNTech		11	–	
Unknown		10	–	
Time from first vaccination (days)		135 (76–210)	99 (66–131)	<0.001

positive for COVID-19. Their demographic and anamnestic information is reported in Table 3. The proportion of individuals who had been vaccinated was higher among the 1046/1251 subjects (83.6%) who tested negative (71.0% vs 49.8%, $p < 0.001$). The median time from the first vaccine dose was 132 days (IQR, 74–207). Of the subjects who tested positive, 137 (66.8%) reported symptoms suggestive of the infection and 131 (63.9%) reported a close contact with individuals diagnosed with COVID-19.

The results of COVID-19 screening by the sniffer dogs were compared with those of the SARS-CoV-2 RT-qPCR test. In this context, inconclusive indications were considered as positive responses for the calculation of test sensitivity and specificity and as incorrect responses when calculating the proportion of correct classifications. The screening performance of each dog is reported in Table 4. Fagan nomograms showing the likelihood ratios and post-test probabilities for positive and negative test results are reported in Fig. 1A.

The interrater agreement among dog pairs is reported in Fig. 1B. It was high between Cloe and the other dogs, between Dayanne and Wave, and between Dayanne and Nenna, and was lower for the other pairs. The overall interrater agreement was 99% (Cohen's $k = 0.873$).

Based on the higher screening performance of Cloe, Dayanne, and Wave (lower limit of sensitivity and specificity 95% CIs >90%) and on their high degree of reciprocal agreement, we calculated the performance of the three pairs resulting from their combination. The data were interpreted as follows: if the two dogs in the pair provided conflicting responses, the response of the pair was recorded as

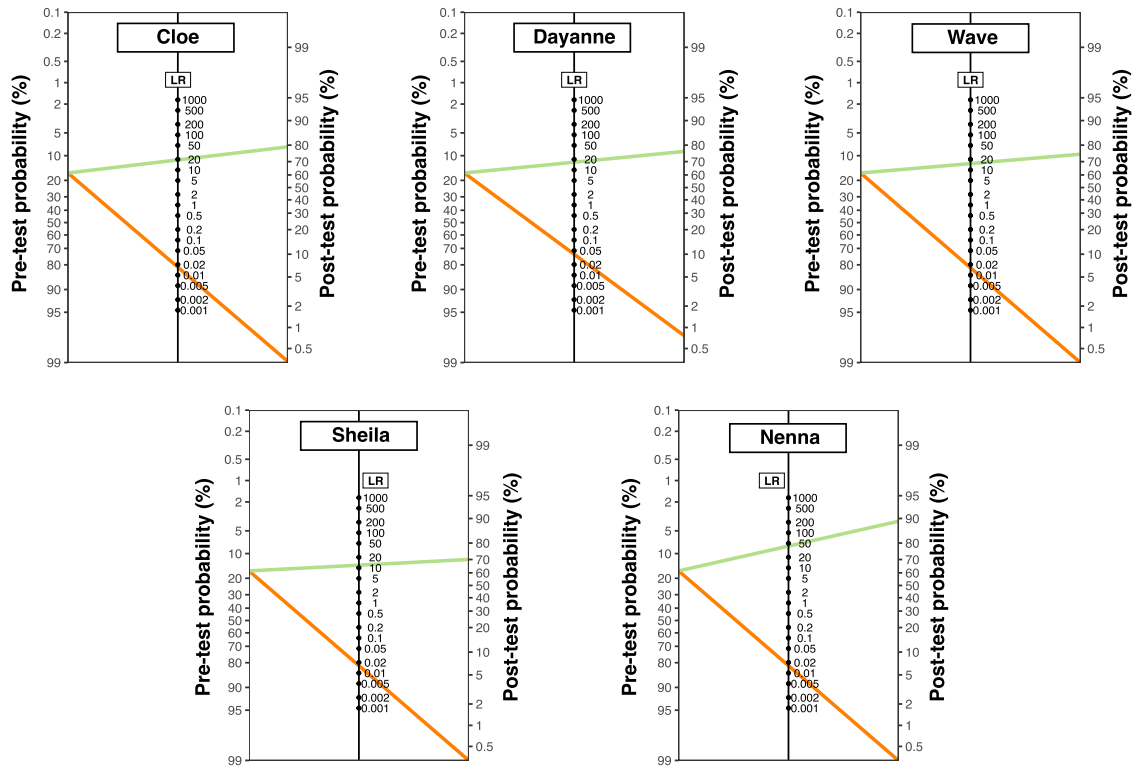
Table 4
Screening performances of the five dogs employed in in vivo COVID-19 screening.

	Cloe	Dayanne	Wave	Shaila	Nenna
No. of subjects tested	1147	1088	363	150	128
True prevalence (%)	16.2	16.3	12.9	4.7	1.6
Apparent prevalence (%)	20.3 (18.1, 22.7)	20.5 (18.1, 23.1)	18.7 (15.1, 23.1)	12.7 (8.3, 18.9)	3.9 (1.7, 8.8)
Sensitivity (%)	98.9 (96.2, 99.7)	96.3 (92.1, 98.3)	100.0 (92.4, 100.0)	100.0 (64.6, 100.0)	100.0 (34.2, 100.0)
Specificity (%)	94.9 (93.3, 96.1)	94.2 (92.4, 95.6)	93.4 (90.1, 95.6)	91.6 (85.9, 95.1)	97.6 (93.2, 99.2)
PPV (%)	79.0 (73.3, 83.7)	76.4 (70.1, 81.7)	69.1 (57.4, 78.8)	36.8 (19.1, 59.0)	40.0 (11.8, 76.9)
NPV (%)	99.8 (99.2, 99.9)	99.2 (98.3, 99.7)	100.0 (98.7, 100.0)	100.0 (97.2, 100.0)	100.0 (97.0, 100.0)
Positive LR	19.4 (14.8, 25.5)	16.6 (12.6, 21.9)	15.0 (10.0, 22.7)	11.9 (6.9, 20.5)	42.0 (13.7, 128.5)
Negative LR	0.01 (0.00, 0.05)	0.04 (0.02, 0.09)	–	–	–
Correct classification (%)	95.20	94.21	94.21	91.33	97.66

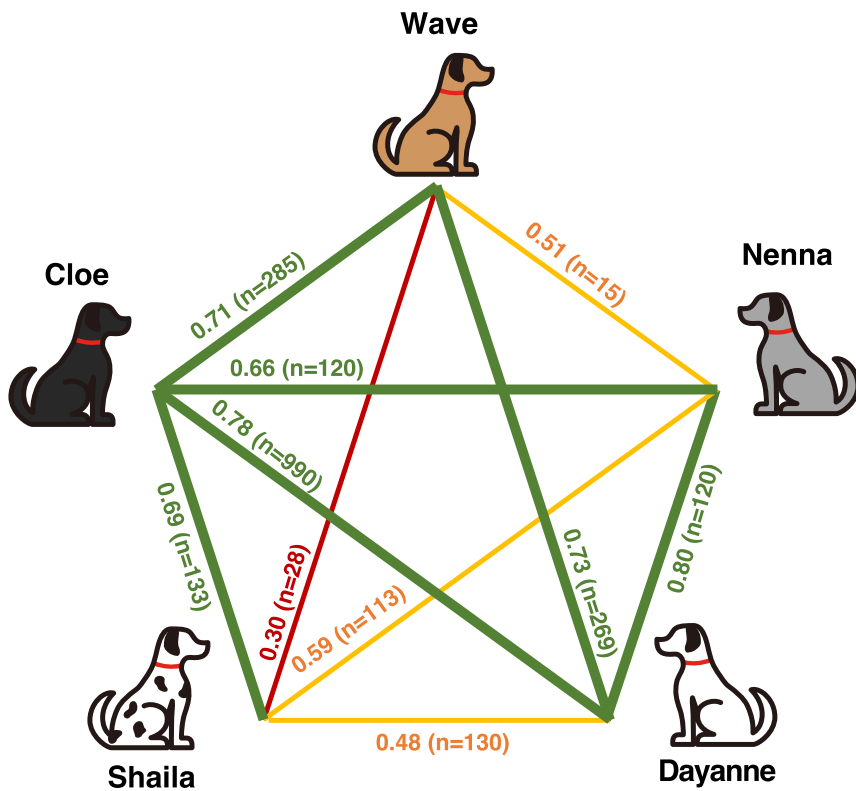
PPV, positive predictive value; NPV, negative predictive value; LR, likelihood ratio. 95% CI intervals in brackets.

The sensitivity of each dog in detecting individuals with COVID-19 infection ranged from 96.3 to 100.0%, whereas specificity ranged from 91.6 to 97.6%; correct classifications ranged from 91.3 to 97.7%. Screening sensitivity and specificity for those subjects who had received at least one dose of COVID-19 vaccine were 98.8–100% and 91.2–98.4%, respectively (Supplementary Table 1).

A



B



(caption on next page)

Fig. 1. (A) Fagan nomogram of each dog: positive (green lines) and negative (red lines) post-test probability with fixed COVID-19 pre-test probability of 16.4%. LR: likelihood ratio. **(B)** Interrater agreement among the dogs (*in vivo* screening). Cohen's kappa and number of screened subjects are reported for each pair. Colors indicate substantial (green), moderate (orange), or fair (red) agreement. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

positive, to enhance sensitivity; in presence of an inconclusive response from one dog, we recorded the response of the other; in case of inconclusive indications from both dogs, the responses were considered as positive for the evaluation of sensitivity and specificity and as incorrect for the evaluation of the proportion of correct classifications. The screening results of the three dog pairs are reported in [Supplementary Table 2](#). Altogether, double screening raised sensitivity up to 100%, whereas specificity ranged from 91.7 to 96.6%. The Cloe-Dayanne pair achieved the highest rate of correct detections (97.17%). The interrater agreement of the three pairs ranged from 95 to 98% and Cohen's kappa from 0.833 to 0.953, reflecting a high degree of agreement among pairs.

Since some key features of the screened individuals had the potential to affect the dogs' responses, we analyzed the age, gender, vaccination status, close contacts with COVID-19-positive subjects and symptoms relatable to COVID-19 of all individuals in relation to the response of each dog ([Table 5](#)). No data are reported for Nenna, because she gave only 3 incorrect indications. Altogether, the proportion of positive SARS-CoV-2 RT-qPCR tests did not differ significantly between correctly and incorrectly classified individuals, although the latter subjects tended to be younger and significant differences in screening performance were found for 2/4 dogs. There were no significant gender-related differences. With regards to vaccination status, the number of subjects who had received at least one vaccine dose was slightly higher among the incorrect responses of 4 dogs and significantly higher among those of the fifth. Notably, for 3/4 dogs the median time from the first vaccination was significantly lower in subjects who were incorrectly identified. Moreover, there was a significantly higher proportion of self-reported symptoms relatable to COVID-19 among the subjects who had been screened incorrectly.

Among the 1046 subjects with a negative nasopharyngeal swab, 107 were incorrectly screened by at least one dog (false positives). Of them, 58 received an inconclusive response by one dog but were correctly identified by the other dog in the pair and were not further considered. The remaining subjects ($n = 49$, median age 46.0 years, 24 males) were either indicated as positive by one ($n = 27$) or two ($n = 16$) dogs or they elicited inconclusive responses from two dogs ($n = 6$). Of these 49 subjects, 42 (85.7%) had been

Table 5
Demographic and anamnestic information of the participants as classified by each dog.

	Correct classifications	Incorrect classifications	p-value
Cloe			
N (%)	1092 (95.2%)	55 (4.8%)	–
Positive SARS-CoV-2 RT-qPCR test (n, %)	180 (16.5%)	6 (10.9%)	0.274
Age (years)	47.0 (32.0–68.0)	42.0 (34.5–54.0)	0.132
Gender (males, %)	466 (42.7%)	27 (48.2%)	0.417
Vaccination with at least one dose (n, %)	726 (66.9%)	44 (78.6%)	0.068
Median time from first vaccination (days)	133 (77–207)	117 (40–171)	0.049
Close contacts with COVID-19-positive individuals (n, %)	494 (45.2%)	30 (54.5%)	0.176
Symptoms relatable to COVID-19 (n, %)	179 (16.4%)	23 (41.8%)	<0.001
Dayanne			
N (%)	1025 (94.2%)	63 (5.8%)	–
Positive SARS-CoV-2 RT-qPCR test (n, %)	169 (16.5%)	11 (17.5%)	0.840
Age (years)	49.0 (34.0–70.0)	45.0 (28.5–55.0)	0.009
Gender (males, %)	444 (43.4%)	26 (40.6%)	0.668
Vaccination with at least one dose (n, %)	695 (68.1%)	50 (78.1%)	0.095
Median time from first vaccination (days)	135 (79–210)	115 (71–154)	0.018
Close contacts with COVID-19-positive individuals (n, %)	473 (46.1%)	41 (65.1%)	0.003
Symptoms relatable to COVID-19 (n, %)	74 (21.6%)	9 (42.9%)	0.025
Wave			
N (%)	342 (94.2%)	21 (5.8%)	–
Positive SARS-CoV-2 RT-qPCR test (n, %)	47 (13.7%)	0	0.069
Age (years)	45.0 (38.0–55.0)	41.0 (38.0–55.0)	0.808
Gender (males, %)	160 (46.8%)	12 (57.1%)	0.356
Vaccination with at least one dose (n, %)	225 (66.0%)	19 (90.5%)	0.020
Median time from first vaccination (days)	121 (77–179)	143 (109–205)	0.164
Close contacts of COVID-19 individuals (n, %)	158 (46.2%)	9 (42.9%)	0.766
Symptoms relatable to COVID-19 (n, %)	74 (21.6%)	9 (42.9%)	0.025
Shailla			
N (%)	137 (91.3%)	13 (8.7%)	–
Positive SARS-CoV-2 RT-qPCR test (n, %)	6 (4.4%)	1 (7.7%)	0.588
Age (years)	84.0 (71.0–90.0)	55.0 (52.0–55.0)	<0.001
Gender (males, %)	47 (34.3%)	9 (69.2%)	0.013
Vaccination with at least one dose (n, %)	126 (92.0%)	12 (92.3%)	0.966
Median time from first vaccination (days)	241 (208–266)	81 (63–115)	<0.001
Close contacts of COVID-19 individuals (n, %)	11 (8.0%)	8 (61.5%)	<0.001
Symptoms relatable to COVID-19 (n, %)	9 (6.6%)	5 (38.5%)	<0.001

Data are median (IQR) or n (%). P values for Mann-Whitney U tests or Chi-square tests of association.

vaccinated (Pfizer-BioNTech, 30; AstraZeneca, 6; Moderna, 4; heterologous AstraZeneca/Pfizer-BioNTech, 1); 21 (42.9%) presented with suggestive symptoms, and 26 (53.1%) reported close contacts with COVID-19-positive subjects. Of note, 9 participants tested positive for COVID-19 up to 48 h before the swab collected at the time of the screening, as reported in the medical report forms, whereas 3 turned positive within 72 h of the COVID-19 test. Moreover, six subjects among those incorrectly indicated as positives suffered from a chronic disease (hypertension, 3; diabetes type 2, 1; asthma, 1; ulcerative colitis, 1).

Of the subjects with a positive nasopharyngeal swab, 10 received an inconclusive response from one of the dogs but were correctly classified as positive by the other and 8 were incorrectly indicated as negative by one of the pair (Dayanne, 6; Cloe, 2). Of these 8 subjects (median age, 32.5 years, 4 males), 6 has not been vaccinated and 2 had received one or 2 doses of the Pfizer-BioNTech vaccine. All 8 of them reported symptoms relatable to COVID-19 and 4 reported close contacts with infected individuals.

Overall, the results of *in vivo* screening on this cohort of 1251 volunteers documented that screening by one dog achieved high sensitivity and specificity and that the combined screening by two dogs achieved an even greater sensitivity.

4.1. Dog behavior and wellness analysis

Statistical analysis of dog behavioral patterns revealed that the validation phase had significantly increased stress levels in dogs ($p = 0.039$), as reflected by changes recorded in displacement activities, attention, olfactory investigation, vocal communicability, submissive behaviors, affiliation, and resting (Fig. 2). On the contrary, conditioning/training and *in vivo* screening at the drive-through center exerted comparable levels of anxiety in dogs, which remain slightly lower than those exhibited in validation. All dogs showed affiliative behavior toward humans and obeyed the commands (e.g., “sniff”). None showed behavioral patterns related to playful behavior toward humans in any task. Only one dog (Shaila) sporadically barked and whined. Overall, dog wellbeing was maintained throughout the study phases.

5. Discussion

Although the vaccination campaign has been successful in significantly reducing the risk of hospitalization for COVID-19, the emergence and rapid spread of new SARS-CoV-2 variants involves a high risk for households, schools, retirement homes, and low-income countries [40–46]. Managing this situation requires an effective contact tracing system and safe, non-invasive, rapid, and cost-effective diagnostic tools. Here, we suggest that scent dogs can provide accurate screening for COVID-19 in such settings.

Even though the use of dogs for disease screening is not universally accepted by the scientific community, a substantial body of evidence demonstrates that dogs can discriminate among biological samples with high sensitivity and specificity. As regards COVID-19, several types of human biological samples have been suggested to undergo a change in the VOC signature that can be detected by properly trained dogs [22–24,26]. Interestingly, in a pilot study in 2016, Angle and coworkers showed that trained dogs can distinguish non-infected cell cultures from cells infected *in vitro* with several viruses, e.g. BVDV (bovine viral diarrhea virus), BHV1 (bovine herpes virus 1), and BPIV3 (bovine parainfluenza virus 3), with very high sensitivity and specificity. They hypothesized that such an ability to discriminate between positive and negative samples might be due to the production of specific VOCs by infected cells and that dogs can be employed as a real-time virus detection tool [47].

Based on these and subsequent reports, we set out to demonstrate that sniffer dogs can provide a viable alternative to traditional screening methods such as rapid antigen testing, which are often invasive, painful, expensive, and laborious, and that it can even

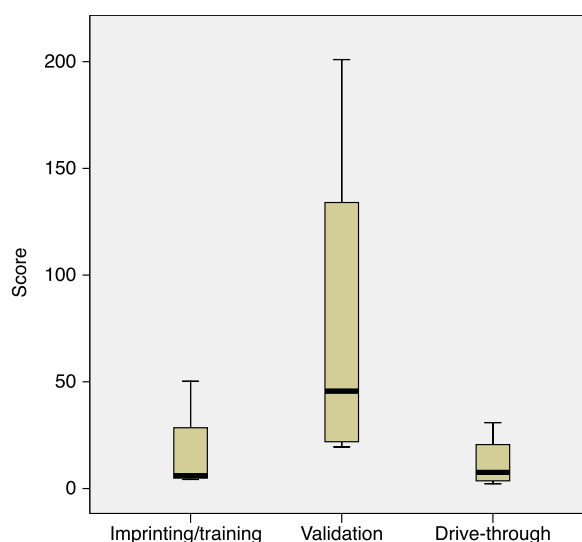


Fig. 2. Behavioral patterns related to comprehensive level of anxiety during imprinting/training, validation, and drive-through screening. Non-parametric data are represented as medians (horizontal bar in the box). The box indicates the interquartile range of 50% of the data.

obviate biological sample collection and processing. Notably, the latter feature entails important additional advantages in terms of savings, *i.e.* time for overworked labs and lab staff, lab material and even lab waste management.

Here, we show that a strict adherence to a protocol of operant conditioning and training with sweat samples, like the one described in the methods, allows to reach the objective of *in vivo* testing with a high sensitivity. Analysis of the *in vivo* screening results of 1251 subjects at the drive-through testing facility showed that the dogs' sensitivity ranged from 96.3 to 100.0% whereas specificity ranged from 91.6 to 97.6%. As an example of individual variation in the detection performance of dogs, Shaila gave five incorrect indications, making her screening less accurate than other dogs. It is difficult to explain these responses, since neither her personality (which is similar to that of the other four dogs) nor her age (she is neither the youngest nor the oldest) set her apart from the other dogs. However, data analysis of these five incorrect indications showed that they were all characterized by a shorter median time from the first vaccination and this probably may have played a role in VOC composition, that Shaila's nose was not able to discriminate.

In addition to the screening performance of each dog, we also examined for the first time the advantage, if any, of having two dogs during the screening session, so that if the dog directly involved in the screening returns a dubious answer, a second dog can be resolute. Since double screening raised sensitivity and NPV up to 100%, we do recommend deploying a dog pair.

Altogether, our protocol, involving dog operant conditioning and training with sweat samples proved highly satisfactory. It is similar to those described by Vesga and colleagues, who used saliva samples for the training phase [28], and by Kantele and co-workers, who used sweat samples for training and travelers' sweat samples collected at an airport for conditioning validation (although this involved setting up a collection center and substantial turnaround times) [29]. Unlike Vesga and colleagues, we found a correlation between incorrect responses and symptom severity; in particular, some dogs gave a greater percentage of incorrect responses for subjects with symptoms relatable to COVID-19 (Cloe and Shaila), for younger individuals (Dayanne and Shaila), and for subjects who had been in close contact with COVID-19-infected patients (Dayanne and Shaila).

Procedure standardization and adherence to the conditioning protocol were ensured throughout the study, as they are prerequisites for the detection of COVID-19 infection even in asymptomatic subjects and in those with mild symptoms, regardless of their vaccination status. Axillary sweat samples were collected using polymer tubes specially devised to absorb VOCs. These tubes are commonly used to train detection dogs and can be stored for weeks at 4 °C (for use within a short period) or at -80 °C for longer-term preservation. Thus, the more than 400 sweat samples that we collected from March to December 2021 and used for operant conditioning and training will be suitable for future training work.

Among the biological samples that can be used for dog operant conditioning, such as breath, tracheobronchial secretions, saliva, and sweat, axillary sweat seemed to be the safest for all the study phases, since molecular RT-PCR analysis has documented that axillary and forehead sweat swabs contain no traces of SARS-CoV-2 [48]. This is all the more relevant since, after several studies showed that dogs are inherently resistant to SARS-CoV-2 [49] and that transmission to other mammals does not occur in experimental conditions [50], cases of human-to-animal transmission have recently been reported, thus raising concern about the potential role of companion animals in the pandemic. According to a large-scale study of SARS-CoV-2 infection in companion animals, 3.3% of dogs and 5.8% of cats had a measurable SARS-CoV-2 neutralizing antibody titer [51,52]. However, as new human variants emerge, their clinical presentation in pets and their role in virus transmission are expected to vary. A recent report has documented the transmission of the Delta variant (B.1.617.2) from vaccinated humans to canines [53]. A community-based study of 119 household dogs in Boston and Idaho, where one or more humans had confirmed SARS-CoV-2 infection, showed that 40% of dogs were seropositive, 5% were PCR-positive, and 21% had clinical signs related to SARS-CoV-2 infection [54]. Similar results have been reported by a Portuguese group [55]. Altogether, these studies suggest that dogs are susceptible to SARS-CoV-2 infection in natural conditions and that although pet-to-human transmission has never yet been described, new reservoirs cannot be excluded due to the virus high mutation rate. Therefore, *in vivo* screening requires that some important safety aspects are addressed in order to minimize the risk of infection: dogs should be kept on a leash and the contact of the nose with the subject to be tested and also their clothing should be avoided. In this way, the test can be safe as well as those previously described and carried out *in vitro* or *ex vivo* contexts [22–26,28,29]. *In vitro* screening using odour samples instead of sniffing real people has some advantages (*e.g.*, bio-safety measures, avoiding dog distraction due to environmental stimuli, no influence of subject's behaviour, possibility of re-tests on the other days), however, *in vivo* screening by trained dogs shows other important benefits: it is rapid, non-invasive as well as economical, since it does not involve actual sampling, it does not take up lab time or resources and requires no waste management, while being suitable to screen large numbers of people. Dog and trainer selection is obviously critical for the performance of animal-assisted screening tools. The dogs employed in our study were selected by experienced dog trainers and veterinary researchers among dogs with a keen sense of smell, an innate propensity to search for biological samples, a set of methodical abilities, and an easy temperament. The latter aspect is actually quite important when dogs are deployed in schools or retirement homes, where the virus spreads very quickly. According to a recent study, properly trained screening dogs experience no stress, but rather find screening a game they enjoy [36].

For these reasons, we also examined the wellbeing of the five dogs. This aspect was never investigated in the field of Covid-19 screening. Analysis of their behavior indicated that they experienced a higher stress level during validation with the helpers compared with the conditioning phase or *in vivo* screening at the drive-through facility. We were surprised, as we thought that the latter task would be more stressful. The cause might lie in the environment rather than the type of work. In fact, conditioning and training were conducted in the same familiar environment, whereas validation was the first of the phases to be conducted in a different place. Nonetheless, all five dogs overcame validation-related stress, since in the next phase, *in vivo* screening at the drive-through, they showed low anxiety levels that were similar to those recorded during training. This suggests that the higher stress experienced during validation, whatever the cause, did not affect their scenting ability, since all dogs successfully went on to *in vivo* screening.

The present study has a number of limitations. First, the different tasks set to the dogs in the conditioning/training and screening phases, *i.e.*, sniffing sweat samples and individuals, respectively, may have involved different VOCs. Secondly, the increasing vaccine

coverage and the spread of new SARS-CoV-2 variants gradually introduce potential confounders that may affect screening performances, although this limitation may be overcome by short refresh sessions. Of note, although we had no data on the genotyping of SARS-CoV-2 variants that have occurred over time, we did not observe a deterioration in the dog performance during all the period of the study (January–December 2021). Thirdly, the study was conducted at a time when the prevalence of COVID-19 was relatively high (16.4% in the cohort). While this circumstance enabled us to achieve the desired statistical power, no definite conclusions can be drawn on the reliability of this screening tool in low-prevalence settings. The need for large population screening arises when prevalence increases. Trained dogs, in settings of low COVID-19 prevalence and therefore reduced activity *in vivo* should be continuously trained, otherwise they would lose the ability acquired with regular training and would not be ready in the case of need. For this reason, a large number of sweat samples (authorized by ethic committees or institutional boards) should be collected and properly stored (−80 °C) for dog training; furthermore, an agreement with the local contact tracing service would be desirable, so that dogs already validated could be also recurrently trained on people (*in vivo*) in a situation where the dog handler knows the outcome of the molecular swab carried out previously by the tested subject.

Altogether, the C19-ScreenDog study confirms the value of *in vivo* screening by carefully trained sniffer dogs, it recommends that two dogs should be deployed to resolve inconclusive responses, and highlights the importance of monitoring dog behavior and wellbeing.

As more data are collected in a wider range of conditions, a consensus may hopefully be reached on the value of employing sniffer dogs to screen people for SARS-CoV-2 as well as other medical conditions, thus providing rapid and economical screening of individuals and even crowds.

Ethic committee approval

This study was approved by the ethical Committees of Regione Marche, CERM (no. 2021/219) and ATS Sardegna (no. 344/2021/CE).

Author contribution statement

Francesca Maria Rosaria Soggiu; Roberto Zampieri: Conceived and designed the experiments; Performed the experiments.
Jacopo Sabbatinelli; Andrea Marchegiani: Analyzed and interpreted the data; Wrote the paper.
Angelica Giuliani; Riccardo Benedetti: Analyzed and interpreted the data.
Francesco Sgarangella; Alberto Tibaldi; Daniela Corsi; Antonio Domenico Procopio; Fabiola Olivieri: Contributed reagents, materials, analysis tools or data.
Sara Calgaro: Performed the experiments; Contributed reagents, materials, analysis tools or data.
Andrea Spaterna: Conceived and designed the experiments.
Maria Rita Rippo: Conceived and designed the experiments; Wrote the paper.

Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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This work is dedicated to the late Serena Zampieri, who daily inspired our work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.heliyon.2023.e15640>.

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