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State of the art, opportunities and challenges in the use of medical detection dogs in the laboratory and in the field

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State of the art, opportunities and challenges in the use of medical detection dogs in the laboratory and in the field

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Editorial: State of the art, opportunities and challenges in the use of medical detection dogs in the laboratory and in the field

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Editorial on the Research Topic
State of the art, opportunities and challenges in the use of medical detection dogs in the laboratory and in the field

In the expansive realm of medical diagnostics, the remarkable olfactory capabilities of canines, combined with their proficiency in operant conditioning, offer a unique avenue for the utilization of medical detection dogs. While the use of scent detection dogs by law enforcement agencies and customs officials to detect substances like money, explosives, or drugs is well-established and widely accepted, the application of medical detection dogs in healthcare is still in its infancy. The recent emergence of research on the employment of medical detection dogs to identify individuals with infectious or non-infectious diseases, particularly during the SARS-CoV-2 pandemic, has garnered significant attention. However, skepticism from the medical profession persists, necessitating a critical exploration of the potential of canine scent detection as a medical test, particularly in the context of infectious diseases such as COVID-19.

The primary objective of this Research Topic is to critically examine the potential of canine scent detection as a medical test, focusing specifically on infectious diseases like COVID-19. By delving into the function and significance of canines' olfaction and evaluating their limitations, as well as their potentials, financial considerations, biosafety, ethical concerns, and animal welfare considerations, a collection of review and expert opinion articles shed light on the role that dogs can play as biomedical detectors for a range of infectious and non-infectious diseases (D'Aniello et al.; Maughan et al.; Meller et al.; Mutesa et al.; Singletary et al.).

One study within this Research Topic focuses on training dogs to detect SARS-CoV-2 positive samples and evaluates their ability to differentiate between SARS-CoV-2 infections and viral infections of a different origin (ten Hagen et al.). The findings reveal that dogs exhibit a mean diagnostic sensitivity of 73.8% and a specificity of 95.1% when presented with swab samples from individuals infected with viruses other than SARS-CoV-2. This demonstrates that dogs can indeed distinguish SARS-CoV-2 infections from other viral infections, although with lower diagnostic sensitivities compared to earlier studies, which used only negative controls as discriminators. The authors conclude that it's necessary to include samples from individuals with other respiratory infectious agents alongside negative and positive COVID-19 samples when training and evaluating the performance of COVID-19 scent detection dogs.

Another study presented in this Research Topic describes a field experience in Mexico where dogs were trained to detect COVID-19 using sweat and saliva samples from positive patients (Mancilla-Tapia et al.). The results indicate that four out of six dogs were able to detect positive samples, demonstrating promising sensitivity and specificity values. The authors suggest that with further exposure to sweat and saliva samples from COVID-19-positive individuals, the dogs' detection capacity could be enhanced, making them valuable allies in pandemic control.

A similar field study was conducted in Rwanda. During the Delta wave, the sensitivity of the dogs' COVID-19 detection ranged from 75.0 to 89.9%, and the specificity from 96.1 to 98.4% for the lowest- and highest-performing dogs, respectively (Mutesa et al.). However, these trained scent detection dogs performed worse during the Omicron wave, with a sensitivity of 36.6 to 41.5%, while specificity remained above 95% for all dogs. This highlights that dogs might need to be retrained for different strains of a virus or that dogs' scent detection performance varies depending on the viral strain. The Rwanda study also pointed out that medical scent detection dogs could be faster and more cost-effective than most antigen or PCR-based tests.

Interestingly, dogs trained to detect acute SARS-CoV-2 infections were also able to detect samples from Long COVID patients (Twele et al.). The dogs reliably detected these Long COVID samples when presented alongside negative samples, but not as reliably when presented alongside acute SARS-CoV-2 samples. The authors suggested that this could be attributed to a titration effect, with Long COVID samples having a lower content of volatile organic compounds (VOCs) than acute SARS-CoV-2 samples.

In another contribution to this Research Topic, researchers explored the use of detection dogs to identify restricted and hazardous biological agents through their volatile organic compound (VOC) signatures (Singletary et al.). The study evaluates the efficacy of a polymer-based training aid in training dogs to detect viral agents, successfully achieving discrimination between agent-based target odors and non-target biological agent-based odors. This highlights the potential of safely utilizing dogs as real-time, mobile detectors in surveillance and screening strategies.

The use of biomedical detection dogs during disease outbreaks, including the ongoing COVID-19 pandemic, is discussed in another article within this Research Topic (Maughan et al.). The authors outline the potential applications, capabilities, and limitations of biomedical detection dogs in disease outbreak scenarios. They also emphasize the need for inter-governmental cooperation and acceptance from the public health community to overcome barriers hindering the implementation of this valuable resource.

Lastly, a comprehensive review article summarizes current evidence and provides a general overview of the diverse aspects that may impact canine medical scent detection (Meller et al.). The experts provide recommendations for the future deployment of medical detection dogs, covering aspects such as the type of dogs, training paradigms, sample characteristics, biosecurity, safety considerations, and training and deployment scenarios. This article can serve as a reference point for the use of medical scent detection in disease control.

The inclusion of dogs as medical detection tools holds significant promise, particularly in the context of infectious diseases, highlighting the One Health approach (1), which recognizes the interconnections between human health, animal health, and the environment, promoting transdisciplinary collaboration. The articles presented in this Research Topic provide valuable insights into the capabilities of medical detection dogs, their potential in disease detection, and the challenges that must be addressed. With continued research and investment in olfactory sciences, future research needs to unlock the full potential of canine scent detection, paving the way for innovative and effective medical testing methods.

In summary, this Research Topic serves as a platform to critically examine the potential of canine medical scent detection, specifically in the context of infectious diseases like COVID-19. By consolidating current knowledge and fostering further research, there is a need to expand our understanding and harness the extraordinary olfactory abilities of canines for the advancement of medical diagnostics, ideally before the next pandemic strikes.

Author contributions

HV: Conceptualization, Writing—original draft. GA: Writing—review and editing. A-LC: Writing—review and editing. DG: Writing—review and editing. FM: Writing—review and editing.

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Conflict of interest

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OPINION
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COVID Sniffer Dogs: Technical and Ethical Concerns

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Hindu medicine showed us that some diseases can alter a humans' scent. Some diseases emit specific volatile organic compounds (VOCs) from exudates, which can be used as a diagnostic tool (1). Recently, occidental culture has started to identify diseases through olfaction, including smallpox (2). Some studies have underlined that humans can identify individuals with bacteria-derived endotoxins through sweat, considering the smell of the sweat unpleasant (3). This olfactory cue could activate a social avoidance, helping to stay far from infected people, thus limiting the contagion in humans, as well as in other animals (4). A human's sense of smell is probably underestimated (5), but it is undoubtedly not capable enough to identify pathogens in people with subliminal changes in odor.

While humans may not be able to detect these subtle odor changes, dogs are capable. They can smell different molecules from the human body released during some emotional states (6–9), and they can be trained to give specific signals when identifying an olfactory signature (i.e., alert signal). Dogs are currently used as special sensors to detect VOCs (10). They have been successfully trained to detect several metabolic conditions and diseases in humans, including hypoglycemia and hyperglycemia (11, 12), epileptic seizures (13), cancers (14), and bacterial and viral infections (15). However, despite the undoubted individual abilities of trained animals, we are still far from a detailed understanding of what exactly the dog responds to and the possibility of generalizing certain abilities to all dogs.

It has been shown in recent studies that dogs can detect people infected with SARS-CoV-2 (16). The use of dogs for this purpose could be critical during emergencies as well as when diagnostic technologies require a long time to be applied. Thus, the dog could be the best detection device in these cases (17) as it represents a faster method of identifying infected people by a non-invasive procedure. Moreover, the use of dogs would allow operators to avoid contact with infected individuals. Though medical detection dogs could be very expensive to train (18), they could test hundreds of people per day, reducing reagent costs.

In this opinion article, we questioned whether there is sufficient scientific support to justify the training and use of dogs as biological detector systems for SARS-CoV-2 in reasonable time frames and safety. To warrant human and dog health, we analyzed the recent scientific literature and discussed different technical and ethical problems with the involvement of dogs for detecting SARS-CoV-2 in people: the "context-shift effect," the overlap of VOC profiles in different diseases and odors that may occasionally co-occur, the procedure to collect samples, and the possible role of the animals as vectors in a zoonotic scenario.

The SARS-CoV-2 pandemic stimulated scientific inquiries on the ability of dogs to recognize the smell of infected human samples. Grandjean et al. (19) trained six detection dogs in recognizing the smell of SARS-CoV-2-infected people by using armpit sweat samples. Their proof-of-concept study concluded that dogs can detect subjects with SARS-CoV-2 with a very high success rate

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(ranging between 76 and 100%). Another scientific study tested the effectiveness of dogs in distinguishing saliva samples and tracheobronchial secretions from SARS-CoV-2 patients with clinical symptoms (20). The authors demonstrated that dogs were able to master the task with high rates of sensitivity (average 82.63%) and specificity (average 96.35%) after a week of training. A third study trained dogs to recognize SARS-CoV-2-infected people from respiratory samples (i.e., saliva, nasopharyngeal swabs or aspirates, and tracheal aspirates) obtained from subjects with mild, moderate, or severe symptomatology (21). Also, in this case, the results were very promising, with the testing procedure reaching 95.5% sensitivity and 99.6% specificity. These studies showed the high efficacy of dogs in recognizing infected people, thus making them useful tools for a very quick screening in crowded places, such as airports and schools. The scientific results have aroused considerable popular enthusiasm, as pointed out by the media, which consider the dogs easy to train and to operate in work contexts.

Besides these advantages, several technical and ethical concerns may occur by using the dogs as sensors of subjects infected by SARS-CoV-2, which seem poorly considered by media and sometimes underestimated in scientific papers. The scientific studies, while demonstrating the effectiveness of the detection dogs for the SARS-CoV-2, have been very cautious in suggesting the use of their results to train dogs for operative purposes, pointing out several pitfalls. Despite that, it seems that the unsolved issues have not reached the practitioners, and therefore, several canine centers are training and using dogs to detect SARS-CoV-2 infected subjects. According to the present scientific evidence, we believe that this approach is not justified at the moment, for several technical and ethical concerns.

The major problem is that no data are available on the performance of dogs in the field as the application of the models in the laboratory studies has not been scientifically tested in work contexts, thus making the effectiveness of dogs questionable. When animals that learned to perform a behavior under a stimulus in a context are moved to a new context, the performance generally drops, which is known as the "context-shift effect" (22) and maybe reflects the loss of information acquired to achieve the goal. This effect has been observed in dogs highly trained for detecting explosives (23). Additionally, a study on detection dogs for lung cancer patients found that by shifting from a hospital to another location, the dogs' performance was significantly reduced, decreasing sensitivity and increasing the occurrence of false positives (24).

The recognition of the VOCs produced by the viral infection presents some difficulties due to the biology of the infection processes, which induce in the host the production of additional VOCs. Generally, viruses do not have their own metabolism; thus, the elicited VOCs could only arise from the inflammatory responses of the infected host (25). It is unknown if SARS-CoV-2 induces changes in VOCs sharing no commonalities with other inflammatory diseases and whether new variants have the same effect in terms of odor changes. Some of the VOCs produced in a single cell line of the infective viruses H9N2, H6N2, and H1N1 appeared selective for each virus, but a plethora of several other non-specific VOCs were present (26). A study on breath analysis using multi-capillary column-ion mobility spectrometry showed that it is possible to discriminate between influenza A and SARS-CoV-2 infections based on the different VOC profiles, although specific VOCs were not identified (27). The authors suggested that dogs could be used to successfully discriminate SARS-CoV-2 infection from other infective diseases. It has been demonstrated that dogs can discriminate VOCs caused by similar virus infections, such as bovine viral diarrhea virus, bovine herpesvirus, and bovine parainfluenza virus (28). Nevertheless, based on previous studies, it is not possible to know for sure if dogs could be confused when detecting between SARS-CoV-2 variants and between variants and other viruses.

In addition to the VOC discrimination problems in infected individuals, another confounding factor could be represented by the overlap of biochemical signals. This phenomenon could confuse dogs, decreasing their detection performance, although the specific combination and concentration of the relevant VOCs may be sufficient for a dog to identify a positive sample. The problem becomes more complex when examining VOCs from the human body while keeping control of the dog's conditioning, which is very important when trying to reduce false positives. For example, two dogs in the bioRxiv version of Grandjean et al. (29) study marked positive a sample from a negative woman that was around the ovulation period, when the luteinizing hormone (LH) peaks. Another study reported that SARS-CoV-2-infected men may show increased levels of LH (30), which makes it plausible to assume that dogs could be conditioned on the metabolic change triggered from the LH instead of that elicited from the virus.

An important factor to be carefully analyzed is the collection and the preparation of the experimental samples for the dog's training. Studies testing the skill of dogs to recognize SARS-CoV-2-infected biological samples worked with a relatively small number of independent and single samples (19–21). This procedure cannot exclude that dogs could memorize the odor of the person, rather than that elicited by the SARS-CoV-2 infection. Indeed, the scientific literature recommends avoiding repeated presentation of samples from the same donors to detection dogs (31).

In the available literature, the samples were collected from symptomatic people; thus, it is unclear whether dogs would alert on samples from asymptomatic individuals. Of course, this is the most important aspect when aiming to identify possible virus spreaders. More research is therefore needed to verify whether dogs could identify asymptomatic and pre-symptomatic individuals. A paper published in August 2020 (32) stated that they were testing dogs to identify asymptomatic people, but the results of this project are not yet available, as well as in the case of Vesga et al. (21).

Beyond the technical aspects of using dogs as sensors, there are also ethical concerns related to the zoonotic transmission of SARS-CoV-2. To date, the bat origin of SARS-CoV-2 remains the most probable cause of the pandemic in humans (33), and several natural, farmed, pet, and wild animal species have

been found infected (34). Minks can have severe symptoms from the infection, and they can die of pneumonia (35). SARS-CoV-2-specific antibodies were not found in 35 animal species tested using double-antigen sandwich ELISA, including dogs and cats (36), but they were detected in dogs and cats by using plaque reduction neutralization tests (37, 38). Dogs were significantly more likely to test positive for SARS-CoV-2-neutralizing antibodies if living in households with infected humans (38, 39), and apart from some negative reports (40), many studies agree that dogs could become infected by humans, although they do not report symptoms from the SARS-CoV-2 infection (41–46). On the other hand, even in healthy humans, most cases were relatively mild or asymptomatic, but older patients and comorbidities could result in severe cases (47). Currently, only a handful of healthy dogs have been studied, and no studies verified the effect of SARS-CoV-2 in old dogs or dogs with other diseases. A study with an artificial infection on five 3-month-old beagles found low susceptibility to SARS-CoV-2 (48), but once again, the samples tested were limited. Should SARS-CoV-2 evolve to be a significant clinical infection in dogs is at the moment unknown. The angiotensin-converting enzyme type 2 receptors (the entry point into cells for some coronaviruses, including SARS-CoV-2) of dogs are very similar to those of humans, with an identity of 83% (49), which does not discharge the risk that dogs could serve as an intermediate host (44, 50). Viruses are well-known to evolve in real time, especially when under immunological pressure, to ease their transmission between humans (51) and from animals to humans (52). A new variant found in humans arose in minks (53). We cannot exclude that new variants in humans may become more infectious for dogs and vice versa, nor can we exclude that new variants in dogs could become more efficient by increasing intraspecies and interspecies transmission. In our opinion, there are currently insufficient results to make sure that dogs could not be or become a reservoir species, whereby we should be more cautious before deliberately exposing dogs to SARS-CoV-2. One of the most important strategies for limiting the pandemic is to identify the potential virus reservoir to prevent any spillover effects, certainly not to facilitate a potential new reservoir species. There is evidence that experimentally infected cats (37, 48, 54), hamsters (55, 56), ferrets (48, 57), and minks (35) may spread SARS-CoV-2, while pigs and some poultry species do not (48, 58, 59). In some cases, the situation is worrying as bilateral transmission between humans and animals has been proved [i.e., minks (60, 61)]. Some studies underlined that there is currently no evidence that infected dogs could be a source of infection for humans (37, 46, 62, 63), although further epidemiological investigations are requested before reaching a definitive conclusion (63). Actually, as a precautionary principle, the fact that there is no scientific evidence does not mean that it could not happen. Some studies have not excluded that dogs could play a role in spreading the virus to other dogs and other animals, including humans (41, 43). The uncertainty of classifying dogs as non-spreaders violates the rules of infection prevention and control.

The authors of the studies that tested dogs to detect people infected with SARS-CoV-2 have been very careful to avoid the exposure of dogs to infections (19, 21, 43), and indeed, in their experimental setting, there was no risk to dogs. However, the laboratory conditions are different from those of the operational work. The fact that SARS-CoV-2 is absent from human sweat (64, 65) may make dogs safe in laboratory tests, but not in a naturalistic scenario where control is more difficult. Although anatomical sites such as armpits are protected by contamination, the part should be uncovered by the hands of the potentially infected subject, which does not warrant sterility, especially when the person is requested to pick up the sample on their own. Fathizadeh et al. (66) collected forehead sweat samples from positive people, and even after disinfecting the skin with 70% ethanol, two positive cases were found in up to 25 infected patients. The authors concluded that although patients' sweat does not contain SARS-CoV-2, it can be easily contaminated. In the study by Jendrny et al. (20), the patient samples were inactivated after incubation for 70–72 h with a chemical compound (i.e., propiolactone) to inactivate the virus. This procedure, while eliminating the risk of contagion, makes faster use of dogs impractical.

To summarize, we reported some suggestions to the problems pointed out in this opinion. Dogs' effectiveness should be tested in different testing environments and naturalistic scenarios to avoid the context-shift effect. It should be a priority to delineate the VOC profiles of the samples of infected people, as collected, using headspace solid-phase microextraction combined with gas chromatography–mass spectrometry, before utilizing them for training the dogs. In the same way, the VOC profiles of the samples should be delineated from non-diseased subjects (67). This procedure would allow comparison of symptomatic and asymptomatic subjects, age classes, sexes, and different parts of the body sample. Although dogs can be trained in the absence of such information, this technical approach is important to allow researchers and stakeholders to control the training at best, thus reaching more suitable performances. The use of VOC-free support materials is recommended to prevent contamination in the results. In the absence of VOC-free gauzes and tubes, these should be pretreated to remove VOC contaminants as described by Cardinali et al. (68). To rule out interindividual differences in body odor, exudates from a large number of different individuals should be collected and mixed (7), or at least different samples should be used for training and testing procedures. To further minimize the chance of dogs memorizing odors from individuals, they should also be trained with the exudates of the same subject collected during both the infective and healthy phases. In that case, it would be necessary to know how long individuals can maintain the odor, especially if matched samples are used. From the reviewed literature, we have a very low chance of SARS-CoV-2 contagion by interacting with our pet dogs. However, it is undoubtful that greater awareness is needed for understanding the possible involvement of dogs in virus hosting and spreading, using a broader vision in the One Health approach. We are not proposing to completely abandon the sniffing dog strategy.

We advocate the precautionary principle and highlight the need for further scientific studies addressing the concerns outlined in this opinion paper before claiming that we can safely use and train dogs effectively to detect SARS-CoV-2-infected people. Particularly, developing a vaccine for dogs could help mitigate the underlined ethical concerns. However, this procedure does not warrant that dogs could serve as a reservoir for the SARS-CoV-2 and develop new variants.

Only after having passed all these scientific steps can we start using dogs in work contexts with more reasonable effectiveness.

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BD'A, CP, MV, MR, PL, and AS have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Discrimination of SARS-CoV-2 Infections From Other Viral Respiratory Infections by Scent Detection Dogs

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Background: Testing of possibly infected individuals remains cornerstone of containing the spread of SARS-CoV-2. Detection dogs could contribute to mass screening. Previous research demonstrated canines' ability to detect SARS-CoV-2-infections but has not investigated if dogs can differentiate between COVID-19 and other virus infections.

Methods: Twelve dogs were trained to detect SARS-CoV-2 positive samples. Three test scenarios were performed to evaluate their ability to discriminate SARS-CoV-2-infections from viral infections of a different aetiology. Naso- and oropharyngeal swab samples from individuals and samples from cell culture both infected with one of 15 viruses that may cause COVID-19-like symptoms were presented as distractors in a randomised, double-blind study. Dogs were either trained with SARS-CoV-2 positive saliva samples (test scenario I and II) or with supernatant from cell cultures (test scenario III).

Results: When using swab samples from individuals infected with viruses other than SARS-CoV-2 as distractors (test scenario I), dogs detected swab samples from SARS-CoV-2-infected individuals with a mean diagnostic sensitivity of 73.8% (95% CI: 66.0–81.7%) and a specificity of 95.1% (95% CI: 92.6–97.7%). In test scenario II and III cell culture supernatant from cells infected

with SARS-CoV-2, cells infected with other coronaviruses and non-infected cells were presented. Dogs achieved mean diagnostic sensitivities of 61.2% (95% CI: 50.7–71.6%, test scenario II) and 75.8% (95% CI: 53.0–98.5%, test scenario III), respectively. The diagnostic specificities were 90.9% (95% CI: 87.3–94.6%, test scenario II) and 90.2% (95% CI: 81.1–99.4%, test scenario III), respectively.

Conclusion: In all three test scenarios the mean specificities were above 90% which indicates that dogs can distinguish SARS-CoV-2-infections from other viral infections. However, compared to earlier studies our scent dogs achieved lower diagnostic sensitivities. To deploy COVID-19 detection dogs as a reliable screening method it is therefore mandatory to include a variety of samples from different viral respiratory tract infections in dog training to ensure a successful discrimination process.

Keywords: canine, volatile organic compound (VOC), COVID-19, screening test, coronavirus, SARS-CoV-2, scent detection dog

INTRODUCTION

The ongoing coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) still affects the life of people all over the world and highlights the need of rapid point-of-care screening tests as a key tool to contain viral spread. The real-time quantitative reverse transcription polymerase chain reaction (rtRT-PCR) is considered the gold standard for diagnosing SARS-CoV-2 infections with high diagnostic accuracy (1), but requires laboratory infrastructure, is time-consuming and can be cost-prohibitive and therefore of limited use for rapid field diagnosis during mass screening in public places, during large events or at ports-of-entry. In these situations, rapid point-of-care antigen tests are used for screening of individuals. However, a recently performed meta-analysis of rapid antigen test application indicates high variability of diagnostic accuracy under real-life conditions, with up to half of asymptomatic patients being tested false negative (2). Medical scent detection dogs could provide an additional screening tool. Several studies have proven canines' extraordinary olfactory acuity to detect individuals with infectious and non-infectious diseases (3). For example, they are capable of detecting a variety of cancer types like lung and breast cancer (4) malaria (5) and bacterial infections caused by *Clostridium difficile* (6), *Staphylococcus aureus* (7), and other bacteria (8). Consequently, several research groups currently train and deploy SARS-CoV-2 detection dogs, recently summarised in a WHO blueprint (9).

In the first published pilot study, dogs were able to detect saliva samples from COVID-19 patients with a diagnostic sensitivity of 83% and specificity of 96% (10), which has now been confirmed by multiple studies with larger sample sets and using the same or different body fluids (sweat or urine) (11–16). Interestingly, dogs are able to transfer their learned scent detection from beta-propiolactone (BPL) inactivated to non-inactivated samples as well as to different body fluids, with comparable diagnostic accuracies indicating a global, specific SARS-CoV-2-associated volatile compound release across different body secretions, independently from the patient being symptomatic or not (17). A recent interesting medical canine scent detection study has used mathematical modelling based on a large cohort of samples from symptomatic and asymptomatic SARS-CoV-2-infections with a wide range of virus loads, represented by varying cycle threshold (Ct) values, to show that these values had a negligible impact on sensitivity compared to lateral flow tests (18). In another recent publication, Hag-Ali and colleagues stated that scent dogs achieved even better sensitivities than the gold standard rtRT-PCR (19). Canine detection is also extremely rapid. Guest et al. report that just two dogs could screen 300 people in 30 min (18). All these studies demonstrate that scent detection dogs can discriminate between samples of SARS-CoV-2-infected, and non-infected healthy individuals with a high level of accuracy and speed. Detection dogs therefore may provide a reliable, fast (2–4 s per sample) screening method for SARS-CoV-2 infections, especially in countries with a lack of access to high-tech screening methods or as a preliminary mass screening for infectious diseases. However, until now, none of the studies evaluated if canines could also distinguish between SARS-CoV-2-infections and infections caused by different human coronaviruses nor other viruses that cause similar symptoms like influenza virus, parainfluenza virus or human rhinovirus. This has been criticised by reviewers (10). Thus, there is an urgent need to test COVID-19 medical scent detection dogs against other respiratory infectious diseases.

A pathogen-specific odour is thought to be detected by dogs being composed of unique volatile organic compounds (VOCs). Laboratory identification of the specific VOC pattern is, however, still in its infancy and there is little published data on the creation of different odours by viral infections. Angle et al. trained dogs to detect cell cultures infected with bovine viral diarrhoea virus (BVDV) (20). After training, these dogs were not just able to discriminate the BVDV-infected cell culture against an uninfected cell culture but also to cell cultures infected by bovine herpes virus 1 (BHV-1) and bovine parainfluenza virus 3 (BPIV-3) achieving high sensitivities and specificities (20). Aksenov and colleagues analysed VOCs emitted from cell cultures infected by different influenza virus subtypes and found unique VOC patterns for each subtype (21). A recent study has

TABLE 1 | Viruses included in our studies.

Virus	Swab sample	Cell culture sample
Severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2)	X	X
Severe acute respiratory syndrome coronavirus type 1 (SARS-CoV-1)		X
Middle East respiratory syndrome-related coronavirus (MERS-CoV)		X
Human coronavirus 229E (HCoV-229E)	X	X
Human coronavirus OC43 (HCoV-OC43)	X	X
Human coronavirus NL63 (HCoV-NL63)	X	X
Human coronavirus HKU1 (HCoV-HKU1)	X	
Influenza A virus subtype H1N1 (A/H1N1)	X	
Influenza A virus subtype H3N2 (A/H3N2)	X	
Influenza B virus subtype Yamagata (B/YAM)	X	
Human respiratory syncytial virus (RSV)	X	
Human metapneumovirus (HMPV)	X	
Human parainfluenza virus type 1 (HPIV-1)	X	
Human parainfluenza virus type 3 (HPIV-3)	X	
Rhinovirus	X	
Adenovirus	X	

analysed breath samples from individuals infected with SARS-CoV-2 or influenza A virus and found also in this experiment virus-specific VOC patterns (22). These studies highlight that not only different virus families but also subtypes within one family have a different odour and could probably be distinguished by scent dogs. The aim of the current study was therefore to demonstrate that medical scent detection dogs can discriminate SARS-CoV-2 infections from other common viral respiratory tract infections, including other coronaviruses.

MATERIALS AND METHODS

Inactivated saliva samples from SARS-CoV-2-infected and healthy individuals as negative controls were used for training. The saliva samples were acquired and prepared as described in previous studies (10). In addition to the set-up of samples in our first study (10), which only included saliva and tracheobronchial secretion samples, the current study included diluted naso- and oropharyngeal swabs and supernatant from cell cultures infected with different respiratory viruses.

Clinical swab samples used in this study were obtained from routine diagnostics at the Robert Koch Institute (Berlin, Germany). For pooled SARS-CoV-2 samples, a mix of 20 naso- and oropharyngeal swabs from PCR confirmed SARS-CoV-2 patients were used, similar Ct values (<1.0 difference) were matched. A 1:3 dilution was performed using swab samples from healthy individuals in phosphate buffered saline (PBS) as a negative matrix. As sample medium, PBS was used for all samples. Furthermore, pooled samples from individuals infected with four different coronaviruses and nine other viruses causing respiratory infections in humans like influenza viruses and parainfluenza viruses were included (**Table 1**). All samples were tested negative via rtRT-PCR for all other viruses included. The status of each included sample was determined by rtRT-PCR (23, 24) at the Centre for Biological Threats and Special Pathogens, Highly Pathogenic Viruses (ZBS1), and Unit 17: Influenza and Other Respiratory Viruses, German National Influenza Centre RKI (Berlin, Germany).

Samples originating from cell culture were derived from the Bundeswehr Institute of Microbiology (Munich, Germany). Six different human coronaviruses were cultured in a human hepatocyte derived carcinoma cell line (HuH7.5 cells, see **Table 1**). Subconfluent to confluent monolayers of HuH7.5 cells were rinsed with serum-free medium and inoculated with SARS-CoV-1 strain "Frankfurt-1" (kindly provided by C. Drosten, Charité Berlin), SARS-CoV-2 strain "BavPat1/2020," (Global Initiative on Sharing All Influenza Data acc. no. EPI_ISL_406862), Middle East respiratory syndrome-related coronavirus (MERS-CoV) strain "EMC" (kindly provided by Bart Haagmans, Erasmus Medical Center Rotterdam), human coronavirus 229E (HCoV-229E) (American Type Culture Collection (ATCC) acc. no. VR-740), HCoV-OC43 (ATCC acc. no. VR-1558) and HCoVNL63. A mock control was included and handled identically with serum-free medium as the inoculum. After incubation for 60 min, the inoculum was discarded, the monolayers were rinsed three times with serum-free medium before supplementing the cells with minimum essential medium (MEM) containing 2% foetal calf serum. Incubation was performed at 33°C, 5% CO_2 and 90% humidity for HCoV-OC43 and -NL63 and at 37°C for the other coronaviruses. Supernatant of the cell cultures was harvested when infection of the monolayers reached 90–100% [assessed either by cytopathic effect or immunofluorescence signal against viral protein (data not shown)]. Supernatants were cleared from cellular debris by centrifugation at 5,000 xg for 10 min in an Eppendorf 5804 centrifuge. Cleared supernatants were inactivated as described in our first study (10). Inactivation success was assessed by lack of growth on Vero E6 cells for SARS-CoV-1, SARS-CoV-2, and

MERS-CoV and inoculation of HuH7.5 cells for HCoV-229E, -NL63, and -OC43. Virus identity and culture success were further confirmed by quantitative RT-PCR (25). Inactivated supernatants were aliquoted and stored at −20°C for storage and at 4°C for subsequent use (**Supplementary Table 1**).

Based on former results showing that BPL inactivation does not change scent dog detection (17) and for easier and safer handling of samples, all samples for either training and testing were BPL inactivated as formerly described (10). Until usage, the samples were deep-frozen at −80°C. For training and testing, a volume of 100 µl per sample was pipetted onto a cotton swab which was placed into a 4 ml glass tube. All samples were handled by the same two persons wearing disposable gloves to prevent odour contamination.

In total, twelve dogs (four males and eight females) were included in our studies. All dogs completed obedience training before the study, and some had a history of scent detection or protection work. Ages ranged between 1 and 5 years. Included dog breeds were Labrador Retriever ($n = 5$), Malinois ($n = 4$), German Shepherd ($n = 2$) and Cocker Spaniel ($n = 1$) (**Supplementary Table 2**).

As described in our previous studies (10, 17) a device called "Detection Dog Training System" (DDTS, Kynoscience UG, Hörstel, Germany) was used for sample presentation and positive reinforcement during training and testing. The DDTS allows for rapid, automatic, randomised, trainer-bias devoid and double-blind sample presentation (10). To verify the recorded results of the DDTS the dogs were filmed during testing and the videos were analysed manually. The training method is based on classical and operant conditioning by using only positive reinforcement as previously described in Jendrny et al. (10, 17). In the present study, the training period lasted 3 days with a high number of sample presentations using inactivated positive saliva samples, or the supernatants of cell cultures infected by SARS-CoV-2 as positive samples. As control samples, negative saliva from healthy individuals (SARS-CoV-2 rtRT-PCR negative) or the supernatant of a non-infected cell culture were utilised. Apart of the "green" dogs used in scenario III, all dogs completed previous training in 2020 for detection of saliva samples of SARS-CoV-2 infected individuals. They were still able to distinguish positive and negative samples even though they had not been trained with SARS-Cov-2 samples for 5 months. After training, the double-blind study was conducted on 2 days using cell cultures and pooled swab samples. In the test scenario I, SARS-CoV-2 positive naso- and oropharyngeal pooled swab samples were utilised as target odours. Distractors were swab samples from patients infected by other viruses causing respiratory tract infections including different coronaviruses (**Table 1**). In the following experiments (test scenario II and III) supernatant from cell cultures infected by several coronaviruses including SARS-CoV-2 and non-infected cell cultures was presented to evaluate if medical scent detection dogs could discriminate between SARS-CoV-2 infection and infection with other coronaviruses or negative controls.

Every nose dip into the DDTS' slots was evaluated with four possible options:

1. True positive (TP): the dog correctly indicates a SARS-CoV-2 positive sample
2. False negative (FN): the dog sniffs shortly at a SARS-CoV-2 positive sample but does not indicate it
3. True negative (TN): the dog sniffs shortly at a negative/distractor sample and correctly does not indicate it
4. False positive (FP): the dog incorrectly indicates a negative/distractor sample

For indicating a sample, the dogs rested with their snout in the respective device test slot ("freezing"). The indication time was recorded by the DDTS and after indicating the target sample the device automatically responded with a reward, i.e., food. Afterwards, the DDTS changed the positions of the presented samples without letting the dog or dog handler know the new positions of negative, distractor or positive samples. This allowed a double-blind sample presentation. In addition, all staff involved was positioned to prevent any interaction or influencing of the animals during the study.

The diagnostic sensitivity as well as diagnostic specificity, positive predictive values (PPV), and negative predictive values (NPV) were calculated according to Trevethan (26). PPV is defined as the probability that people with a positive screening test result indeed do have the condition of interest and was calculated as [true positive/(true positive + false positive)] × 100. NPV is defined as the probability that people with a negative screening test result do not have the condition of interest and was calculated [true negative/(false negative + true negative)] × 100. 95% confidence intervals (CIs) for sensitivity, specificity, PPV, and NPV were calculated with the hybrid Wilson/Brown method (27). Means of sensitivity, specificity, PPV, NPV, and accuracy with corresponding 95% CIs were also calculated per scenario. Two-tailed Fisher's exact test was used for analysis of the contingency tables; a $P \leq 0.05$ was considered significant. All calculations were done with the Prism 9 software from GraphPad (La Jolla, CA, USA).

This study was carried out in accordance with the ethical requirements established by the Declaration of Helsinki. The study obtained ethical approval by the Berlin Chamber of Physicians (Eth 20/40) and was approved by the local Ethics Committee of Hannover Medical School (MHH) and Hamburg Medical Association for the University Medical-Center Hamburg-Eppendorf (UKE) (ethic consent number 9042_BO_K_2020 and PV7298, respectively). Written informed consent from all participants was obtained before sample collection. Animal work according to the study protocol and design was approved by the German Armed Forces.

RESULTS

Three test scenarios were performed to address the aim of the study. In the first test scenario (scenario I), dogs who were trained with saliva samples discriminated between SARS-CoV-2 swab samples and swab samples from patients infected by other respiratory tract infection viruses (**Table 1**). The dogs achieved a mean sensitivity of 73.8% (95% CI: 66.0–81.7%) and a specificity of 95.1% (95% CI: 92.6–97.7%). In the following test scenario

TABLE 2 | Diagnostic performance of the scent detection dogs.

Test scenario	Dog	Detection	SARS-CoV-2 infection status		Total number of sample presentations	Diagnostic specificity (Sp)	Diagnostic sensitivity (Se)	Confidence interval (95% CI) Sp	Confidence interval (95% CI) Se	Positive predictive value (PPV)	Negative predictive value (NPV)	Confidence interval (95% CI) PPV	Confidence interval (95% CI) NPV	Accuracy	Fisher's exact test *p*-value
			Positive	Negative/other pathogen											
I) Swab samples (after training with inactivated saliva samples)	Dog 1	Yes	16	8	132	0.926	0.667	0.861–0.962	0.467–0.82	0.667	0.926	0.467–0.82	0.861–0.962	0.879	<0.0001
		No	8	100											
	Dog 2	Yes	19	6	154	0.954	0.826	0.904–0.979	0.629–0.93	0.76	0.969	0.566–0.886	0.923–0.988	0.935	<0.0001
		No	4	125											
	Dog 3	Yes	16	6	139	0.948	0.667	0.891–0.976	0.467–0.82	0.727	0.932	0.518–0.868	0.871–0.965	0.899	<0.0001
		No	8	109											
	Dog 4	Yes	17	12	143	0.899	0.708	0.832–0.941	0.508–0.851	0.586	0.939	0.407–0.745	0.879–0.97	0.867	<0.0001
		No	7	107											
	Dog 5	Yes	18	7	154	0.946	0.75	0.893–0.974	0.551–0.88	0.72	0.953	0.524–0.857	0.902–0.979	0.916	<0.0001
		No	6	123											
	Dog 6	Yes	15	0	164	1	0.6	0.973–1	0.407–0.766	1	0.933	0.796–1	0.881–0.963	0.939	<0.0001
		No	10	139											
	Dog 7	Yes	18	4	122	0.96	0.818	0.902–0.984	0.615–0.927	0.818	0.96	0.615–0.927	0.902–0.984	0.934	<0.0001
		No	4	96											
	Dog 8	Yes	20	3	146	0.976	0.87	0.931–0.993	0.679–0.955	0.87	0.976	0.679–0.955	0.931–0.993	0.959	<0.0001
		No	3	120											
						Mean Sp	**Mean Se**	**95% CI of mean Sp**	**95% CI of mean Se**	**Mean PPV**	**Mean NPV**	**95% CI of mean PPV**	**95% CI of mean NPV**	**Mean accuracy**	**95% CI of mean accuracy**
						0.951	0.738	0.926–0.977	0.66–0.817	0.768	0.948	0.662–0.875	0.933–0.964	0.916	0.889–0.943
II) Cell culture samples (after training with inactivated saliva samples)	Dog 1	Yes	8	6	75	0.905	0.667	0.807–0.956	0.391–0.862	0.571	0.934	0.326–0.786	0.843–0.974	0.867	<0.0001
		No	4	57											
	Dog 2	Yes	4	3	53	0.933	0.5	0.821–0.977	0.215–0.785	0.571	0.913	0.25–0.842	0.797–0.966	0.868	0.0068
		No	4	42											
	Dog 3	Yes	7	7	65	0.865	0.538	0.747–0.933	0.291–0.768	0.5	0.882	0.268–0.732	0.766–0.945	0.8	0.0042
		No	6	45											
	Dog 4	Yes	8	2	79	0.97	0.615	0.896–0.995	0.355–0.823	0.8	0.928	0.49–0.964	0.841–0.969	0.911	<0.0001
		No	5	64											
	Dog 5	Yes	9	2	45	0.941	0.818	0.809–0.99	0.523–0.968	0.818	0.941	0.523–0.968	0.809–0.99	0.911	<0.0001
		No	2	32											
	Dog 6	Yes	8	11	75	0.82	0.571	0.705–0.896	0.326–0.786	0.421	0.893	0.231–0.637	0.785–0.95	0.773	0.0051
		No	6	50											
	Dog 7	Yes	7	2	51	0.946	0.5	0.823–0.99	0.268–0.732	0.778	0.833	0.453–0.961	0.694–0.917	0.824	0.0008
		No	7	35											
	Dog 8	Yes	10	5	81	0.928	833	0.841–0.969	0.552–97	0.667	0.97	0.417–0.848	0.896–0.955	0.914	<0.0001
		No	2	64											
	Dog 9	Yes	6	7	69	0.875	0.462	0.764–0.938	0.232–0.709	0.462	0.875	0.232–0.709	0.764–0.938	0.797	0.0119
		No	7	49											
						0.909	0.621	0.873–0.946	0.507–0.716	0.621	0.908	0.505–0.737	0.876–0.939	0.852	0.81–0.894
						Mean Sp	**Mean Se**	**95% CI of mean Sp**	**95% CI of mean Se**	**Mean PPV**	**Mean NPV**	**95% CI of mean PPV**	**95% CI of mean NPV**	**Mean accuracy**	**95% CI of mean accuracy**
						0.951	0.738	0.926–0.977	0.66–0.817	0.768	0.948	0.662–0.875	0.933–0.964	0.916	0.889–0.943
III) Cell culture samples (after training with cell culture samples)	Dog 1	Yes	10	6	44	0.818	0.909	0.656–0.914	0.623–0.995	0.625	0.964	0.386–0.815	0.823–0.998	0.841	<0.0001
		No	1	27											
	Dog 2	Yes	10	0	30	1	1	0.839–1	0.722–1	1	1	0.722–1	0.839–1	1	<0.0001
		No	0	20											

(Continued)

TABLE 2 | Continued

Test scenario	Dog	Detection	SARS-CoV-2 infection status		Total number of sample presentations	Diagnostic specificity (Sp)	Diagnostic sensitivity (Se)	Confidence interval (95% CI) Sp	Confidence interval (95% CI) Se	Positive predictive value (PPV)	Negative predictive value (NPV)	Confidence interval (95% CI) PPV	Confidence interval (95% CI) NPV	Accuracy	Fisher's exact test *p*-value
			Positive	Negative/other pathogen											
	Dog 3	Yes	7	10	78	0.851	0.636	0.747–0.917	0.354–0.848	0.412	0.934	0.216–0.64	0.843–0.974	0.821	0.0014
		No	4	57											
	Dog 4	Yes	9	3	76	0.951	0.6	0.865–0.987	0.357–0.802	0.75	0.906	0.468–0.911	0.81–0.956	0.882	<0.0001
		No	6	58											
	Dog 5	Yes	9	7	79	0.892	0.643	0.794–0.947	0.388–0.837	0.563	0.921	0.332–0.769	0.827–0.966	0.848	<0.0001
		No	5	58											
						Mean Sp	**Mean Se**	**95% CI of mean Sp**	**95% CI of mean Se**	**Mean PPV**	**Mean NPV**	**95% CI of mean PPV**	**95% CI of mean NPV**	**Mean accuracy**	**95% CI of mean accuracy**
						0.902	0.758	0.811–0.994	0.53–0.985	0.67	0.945	0.396–0.944	0.899–0.991	0.878	0.79–0.967

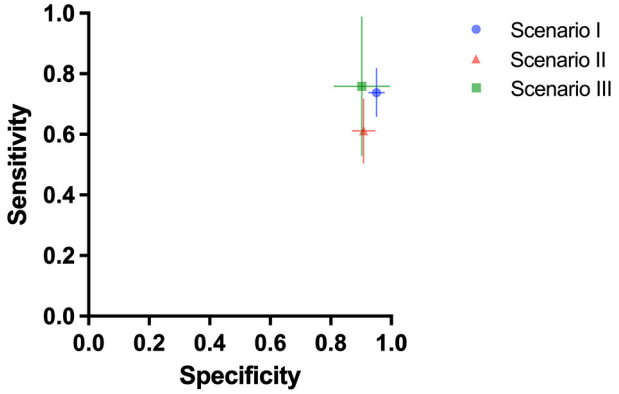

FIGURE 1 | Mean diagnostic specificity and sensitivity for all dogs for swab samples with preceding saliva training (test scenario I: blue circle), cell culture samples with preceding saliva training (test scenario II: red triangle), and cell culture samples with preceding cell culture training (test scenario III: green square), respectively. The 95% confidence intervals for specificity and sensitivity are shown with horizontal and vertical lines, respectively.

(scenario II) cell culture supernatants from cells infected by different coronaviruses or non-infected cells were comparatively presented to the dogs. Dogs were able to discriminate the SARS-CoV-2 supernatant from supernatants from other coronaviruses and non-infected controls with a mean sensitivity of 61.2% (95% CI: 50.7–71.6%) and a specificity of 90.9% (95% CI: 87.3–94.6%). In the last test scenario (scenario III), not formerly trained ("green") dogs were directly trained using the supernatant from cell cultures infected by SARS-CoV-2. The dogs achieved a mean sensitivity of 75.8% (95% CI: 53.0–98.5%) and a specificity of 90.2% (95% CI: 81.1–99.4%) (**Table 2**; **Figure 1**).

Overall, a total of 2,054 sample presentations were performed in three different test scenarios. During the presentation of swab samples (test scenario I), 139 correct indications and 50 false rejections of SARS-CoV-2 positive swab samples were recorded, while 919 correct rejections and only 46 false indications of swab samples from the other 13 viruses were made. When the supernatants of the different cell cultures were presented to the dogs trained with saliva (test scenario II), they indicated 67 SARS-CoV-2 sample presentations correctly and rejected 43 positive samples incorrectly. They made 45 false positive responses to any one of three other coronaviruses or one non-infected control and 443 correct rejections. In comparison, dogs trained with cell cultures for 3 days (test scenario III) correctly identified 45 SARS-CoV-2 supernatants correctly but incorrectly rejected 16 positive samples, whereas 220 correctly negative and 26 false positive responses to the other seven cell culture samples (six different coronaviruses and one non-infected control) were made (**Figure 2**).

DISCUSSION

Rapid detection of SARS-CoV-2 infections remains one of the main strategies to control the current global pandemic. Several studies have shown that trained scent detection dogs

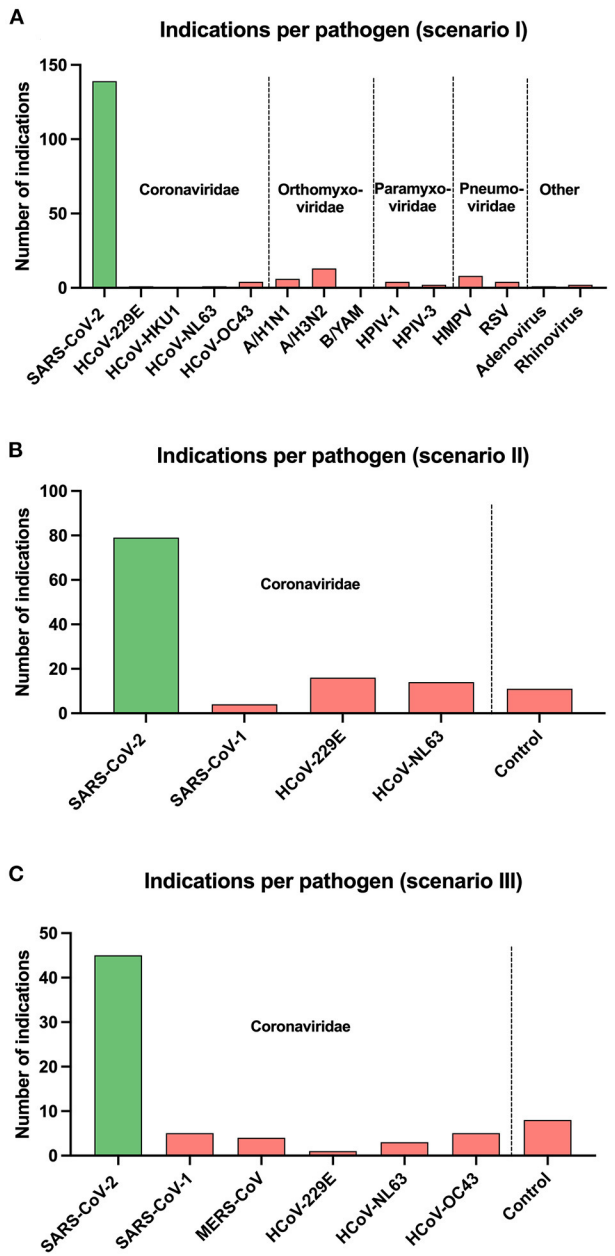

FIGURE 2 | Overall number of indications per pathogen for each test scenario of scent dogs is shown as vertical bars: **(A)** test scenario I (swab samples with preceding saliva training), **(B)** test scenario II (cell culture samples with preceding saliva training), and **(C)** test scenario III (cell culture samples with preceding cell culture training). Pathogens are grouped on the x-axis according to their virus families and separated by vertical dotted lines. *Coronaviridae*: severe acute respiratory syndrome coronavirus 1 and 2 (SARS-CoV-1 and SARS-CoV-2), Middle East respiratory syndrome-related coronavirus (MERS-CoV), human coronavirus 229E (HCoV-229E), human coronavirus HKU1 (HCoV-HKU1), human coronavirus NL63 (HCoV-NL63), human coronavirus OC43 (HCoV-OC43). *Orthomyxoviridae*: influenza A virus subtype H1N1 (A/H1N1), influenza A virus subtype H3N2 (A/H3N2), influenza B virus subtype Yamagata (B/YAM). *Paramyxoviridae*: human parainfluenza virus type 1 (HPIV-1), human parainfluenza virus type 3 (HPIV-3). *Pneumoviridae*: human metapneumovirus (HMPV), respiratory syncytial virus (RSV).

can discriminate SARS-CoV-2-infected individuals from healthy individuals with high diagnostic accuracies. However, to use medical detection dogs, as a reliable diagnostic test, it is important to ensure their capability to distinguish between samples from SARS-CoV-2-infected people and other viral infections causing similar symptoms. In the current study, dogs were able to discriminate samples from SARS-CoV-2 infected individuals and cell cultures from those infected with one of 15 other common acute respiratory viruses as distractors.

During 964 presentations of swab samples from patients with different viral infections other than SARS-CoV-2 (test scenario I), the scent dogs alerted falsely to 46 samples, which led to a mean specificity of 95.1%. This specificity is comparable to our first pilot study by Jendrny et al. in which we only used samples from healthy, SARS-CoV-2 negative individuals without any respiratory symptoms as control samples and obtained a specificity of 96.35% (10). When using cell culture samples (test scenario II and III), the canines achieved mean specificities of 90.2% and 90.6%, respectively. Although the diagnostic specificities were found lower compared to our previous studies (10, 17), our results indicate that dogs can distinguish SARS-CoV-2 from other viral respiratory infections.

When presenting cell culture samples to dogs trained by saliva, the mean sensitivity was 61.2%, which is lower compared to sensitivities ranging from 82% to 95% in our previous studies (10, 17). The reduced performance in some dogs could be explained by not entirely identical VOC pattern that is released from cell culture supernatant compared to VOCs originating from saliva samples of the organism, the human body. In contrast to this observation, some of the dogs were able to directly transfer from saliva samples to supernatant samples. Possible explanations for this inconsistency might be that not every dog is conditioned to exactly the same VOC pattern or that individual dogs may not recognise identical VOC patterns. It is unknown which disease-specific VOCs are detected by the dogs and it is reasonable that the dogs learned slightly different VOC patterns as positive. Despite this, results from previous studies demonstrate that all dogs could be successfully trained to a disease-specific odour (10, 17). It would have been best if we had used saliva samples from individuals with different viral infections for testing. Unfortunately, this was not possible, as there were currently few infections with other respiratory viruses due to the high hygiene standards established during SARS-CoV-2 pandemic to prevent virus transmission and spread. Cell cultures provide the opportunity to generate odour samples independent of the availability of acute patients and it is possible to include samples of a wide range of different viruses, including other coronaviruses. Therefore, we decided to directly train five dogs with cell culture supernatant from human cells infected by SARS-CoV-2 instead of saliva and assess their diagnostic performance in a subsequent session. After just 3 days of training the mean sensitivity increased from 61.2% for the dogs trained with saliva samples to 75.8% for the five dogs trained with SARS-CoV-2 cell culture supernatant. A longer training period likely would have increased diagnostic accuracies (20).

The prevalence in our study was 17.5%. The high prevalence is a result of the fact that we presented one SARS-CoV-2 positive sample next to several samples from different viruses. In a pandemic, prevalences will vary and are usually significantly lower. When the prevalence falls below a certain threshold the dogs might get frustrated and stop searching for a positive sample. To ensure that scent dogs keep their diagnostic accuracies in case of a low prevalence and to have an internal control it is important to reward dogs during a test run not only for finding positive samples but also for not indicating wrongly negative samples. Furthermore, one has to ensure that the training entails an increasing number of empty runs (presentation of only negative samples). Dogs' frustrations levels should then be recorded and considered when working in the field, ensuring dogs will receive a sufficient number of rewards to keep them positively engaged in the field. A further limitation can be the dog handler. Not only the dog but also the dog's handler has an individual character, an individual training level and therefore require different training requirements. The DDTS can overcome some of these limitations, as dogs can be trained to work independently [**Supplementary Video 1** and see Jendrny et al. (10)].

The prevalence of an infectious diseases is dynamic in a pandemic. As aforementioned the prevalence in our test paradigm was higher than in the current pandemic situation, which is further subject to change with an ever-increasing number of people getting vaccinated. The real positive predictive values would be with the current prevalence lower when sensitivity and specificity of dogs remain unchanged and the dogs would be deployed without a rewarding system. However, as in our test setting, a lower prevalence does not impact the performance of the dogs necessarily, as the frequency of getting rewarded is above the prevalence of the disease.

The disease-specific odour that can be detected by dogs is thought to be determined by a specific pattern of VOCs. VOCs are produced by cell metabolism and released with breath, urine, saliva, blood, faeces, sweat and other body fluids (28). In comparison to bacteria, viruses have no own metabolism, but the common hypothesis is that viruses can change the metabolism of the infected host cell and therefore determine a special VOC pattern (29). The composition of emitted VOCs in human body fluids are not only a result of non-infectious and infectious diseases but depend on a variety of factors such as age, sex, and diet (30). Every human, regardless of an infection, emits a variety of VOCs in a special pattern, which is called the human volatilome, and this pattern determines the unique body odour (28, 31). The usage of cell cultures provides the opportunity to exclude a lot of these influencing factors. Human samples contain a wide array of virus-independent VOCs and dogs need to seek out the disease-specific odour. When training with samples from individuals it is necessary to include a large number of human subjects to ensure that dogs are conditioned on the disease-specific odour. Training with SARS-CoV-2-infected cell cultures as the target odour and an equally treated, non-infected cell culture as negative control sample possibly simplifies the discrimination process for the dog between infected and non-infected samples. For our studies HuH7.5, a well differentiated cell line, was utilised to produce samples from different coronavirus infections and a non-infected cell culture as control. HuH7.5 cells were originally derived from a liver tumour in a 57-year-old Japanese male in 1982. HuH7.5 are particularly used for propagating the hepatitis C virus *in vitro* (32) and provided the opportunity to cultivate six different coronaviruses in the same human cellular background. For all cell culture samples the same serum-free medium and MEM containing 2% foetal calf serum were used to prevent odour interferences. Therefore, the odours of the cell culture samples were not affected by individual factors of the host like age, sex, diet or underlying medical conditions which are usually influencing odour samples. Consequently, the differences in the emitted VOCs between the infected cell culture samples are based on the specific coronavirus. However, cell culture samples do not take into account that during the infection a lot of changes occur in the infected organs and organism, like the complex mix of inflammatory reaction and cellular influx with specific se- and excretion and debris of dying cells. Obviously, this is not mimicked in a cell culture and may at least in part explain some of the discrepancies between swab sample and cell culture results. Consequently, when training for real-life deployment samples from infected individuals should be preferred. However, regardless the complex changes in VOC profile in infected individuals our cell culture results indicate that the VOCs created by infected cells are virus specific, which is why scent dogs can discriminate cells infected with different viruses. Furthermore, apart from scenting VOC patterns, dogs might also be capable of detecting directly viruses or viral proteins via their vomeronasal organ. The vomeronasal organ is capable of processing a wide variety of molecules, including proteins, thus representing a different and additional mechanism of odour perception (33, 34), which could explain dogs being able to discriminate specific viruses.

Several studies have evaluated the VOCs of viral infections (21, 35–37). It has been shown, that human tracheobronchial epithelial (TBE) cells infected with human rhinovirus (HRV) emit distinct VOCs compared to non-infected cells and cells inoculated with inactivated HRV (38). In a follow-up study, TBE cells were infected with HRV or influenza A virus subtype H1N1 (A/H1N1) with corresponding non-infected controls. Emitted VOCs were analysed via gas chromatography-mass spectrometry. Fifty-four unique VOCs were found distinguishing virus-infected from -uninfected cells. Forty of these VOCs were specific for A/H1N1-infected cells, but five occurred in A/H1N1- and HRV-VOC patterns (35). In addition, infections by different influenza A virus subtypes result in disparate VOC patterns (21). Current data from several studies suggest that SARS-CoV-2 infections create a specific VOC pattern which could be used in diagnostics (22, 37, 39, 40). Steppert et al. analysed exhaled breath from persons infected by SARS-CoV-2 or influenza A virus and healthy people via multi-capillary column-ion mobility spectrometry. They were able to discriminate between SARS-CoV-2, influenza A virus and controls in a few minutes which indicates that SARS-CoV-2 and influenza A virus infections can be distinguished by their differing VOC patterns (22). In summary, these data indicate that every viral

infection creates its own specific VOC pattern and can therefore be discriminated.

Preliminary work on discriminating different viral infections by scent dogs was performed by Angle et al. (20). They used cell cultures infected with BVDV as the target sample and cell cultures infected with BHV-1 or BPIV-3 as distractors presented in a scent wheel with eight arms. After a training period of 2 months, the two dogs achieved sensitivities of 85% and 96.7% and specificities of 98.1% and 99.3% (20). These results indicate that trained dogs can discriminate different viral infections by their odour which is in accordance with our findings. A successful discrimination is fundamental in scent detection training, meaning to be able to differentiate the target odour from similar odours (41). The main aim of our study was to prove that dogs can discriminate different viral respiratory tract infections by their odour. In all three test scenarios our dogs achieved mean specificities above 90% which indicates their capability to distinguish SARS-CoV-2 infections from infections with other viruses. However, in contrast to earlier findings our scent dogs achieved lower diagnostic accuracies (10, 17). This discrepancy could be attributed to a more similar odour of SARS-CoV-2 infections to other viral infections than to non-infected individuals or cells. The similar odour of different viral infections probably resulted in a lack of discrimination and should be considered in subsequent training and testing. Our study clearly shows that it is mandatory to include other viruses in dog training to keep the diagnostic accuracy high. Our results indicate that presenting samples from different viral infections in the early training phase would improve the dogs' diagnostic skills and will support a successful discrimination process. This would ensure that scent dogs are conditioned to the unique smell of a SARS-CoV-2 infection and not to additional VOCs which are produced by several viral infections.

CONCLUSION

In the current situation rapid antigen tests are used for screening people for SARS-CoV-2 infections, which generate test results within 15 min. Manufacturers state sensitivities above 90% (42), but several studies determined significantly lower sensitivities with certain tests (2, 43). The WHO and the Paul Ehrlich Institute (PEI, Langen, Germany) recommend a sensitivity of ≥80% and a specificity of ≥97% for rapid antigen tests (44, 45). In real life settings while screening asymptomatic people, Dinnes et al. found a mean sensitivity of 58% (2). For their deployment as a reliable diagnostic test COVID-19 detection dogs should meet the criteria recommended by the WHO and national institution like PEI. Previous studies indicate that the scent dog method could meet these criteria (14, 17, 19). In several studies dogs showed their capability to distinguish SARS-CoV-2 positive samples from negative samples with high diagnostic accuracy regardless of training method or sample type (11, 12, 14–19). Our results demonstrate their ability to differentiate viral respiratory tract infections by their odour but suggest including a variety of viruses during dog training to guarantee a high diagnostic accuracy. Further research should be performed to validate dogs' scent recognition capabilities as diagnostic tool, especially in asymptomatic or pre-symptomatic patients, vaccinated or not, as infected individuals spread virus and could even be super-spreaders, as has been documented (46). Follow-up study in this category is needed by testing larger cohort of swabs from asymptomatic rtRT-PCR positive individuals.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Berlin Chamber of Physicians (Eth 20/40) Hannover Medical School 9042_BO_K_2020 University Medical-Center Hamburg-Eppendorf PV7298. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by German Armed Forces.

AUTHOR CONTRIBUTIONS

NH participated in the planning of the study, carried out the main practical work, data analyses, and helped draught the manuscript. FT, SM, PJ, and HV designed and coordinated the study, drafted the manuscript (FT), conducted and coordinated (FT) the sample acquisition, and were responsible for data analyses (SM). MK-B, CS, and AO participated in the planning of the laboratory part of the study and were in charge for the legal permission for sample processing. CS, RE, KZ, and RW carried out the laboratory work at the Research Center for Emerging Infections and Zoonoses (CS) and Bundeswehr Institute of Microbiology (RE, KZ, and RW) including sample preparation, virus inactivation, and RtRT-PCR. HE programmed the DDTS software and supported the dog training. IP, TW, MM, AF, and MA were in charge for the ethical approval, patient recruitment and sample collection (IP and AF) at Hannover Medical School (IP, TW, and MM), and University Medical-Center Hamburg-Eppendorf (AF and MA). ANi and AP coordinated the sample acquisition at the Robert Koch Institute. AP, JM, and EK performed the sample preparation including rtRT-PCR at the Robert Koch Institute. ANa and EP were responsible for coordinating the acquisition and data of samples as part of the support provided by the German armed forces. CE was responsible for the special research proposal of the German Armed Forces as project manager on the part of the German Armed Forces. ME coordinated the cooperation with the University of Veterinary Medicine Hannover. AB participated in the planning of the cultivation of the different coronaviruses and provided the HuH7.5 cells. ES was responsible for the dog training and helped with data analyses. ME and ES were also involved in designing and coordinating the study. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.749588/full#supplementary-material

Supplementary Table 1 | Characteristics of the samples used for the study.

Supplementary Video 1 | Scent detection dog working with Detection Dog Training System (DDTS). The video shows the Malinois "Filou" during a detection session. At each detection run only one hole is presenting the target scent, in this case SARS-CoV-2 samples, with the other six holes presenting the distractors. The dog indicates a positive detection by remaining with the nose longer than 2 s in the hole. This will release a food reward, a beeping sound and will be recorded automatically. The device then rearranges the sample presentation automatically and randomly.

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Dogs Detecting COVID-19 From Sweat and Saliva of Positive People: A Field Experience in Mexico

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Context: Molecular tests are useful in detecting COVID-19, but they are expensive in developing countries. COVID-19-sniffing dogs are an alternative due to their reported sensitivity (>80%) and specificity (>90%). However, most of the published evidence is experimental, and there is a need to determine the performance of the dogs in field conditions. Hence, we aimed to test the sensitivity and specificity of COVID-19-sniffing dogs in the field.

Methods: We trained four dogs with sweat and three dogs with saliva of COVID-19-positive patients, respectively, for 4.5 months. The samples were obtained from a health center in Hermosillo, Sonora, with the restriction to spend 5 min per patient. We calculated sensitivity, specificity, and their 95% confidence intervals (CI).

Results: Two sweat-sniffing dogs reached 76 and 80% sensitivity, with the 95% CI not overlapping the random value of 50%, and 75 and 88% specificity, with the 95% CI not overlapping the 50% value. The 95% CI of the sensitivity and specificity of the other two sweat dogs overlapped the 50% value. Two saliva-sniffing dogs had 70 and 78% sensitivity, and the 95% CI of their sensitivity and specificity did not overlap the 50% value. The 95% CI of the third dog's sensitivity and specificity overlapped the 50% value.

Conclusion: Four of the six dogs were able to detect positive samples of patients with COVID-19, with sensitivity and specificity values significantly different from random in the field. We considered the performance of the dogs promising because it is reasonable to expect that with gauze exposed for a longer time to sweat and saliva of people with COVID-19, their detection capacity would improve. The target is to reach the sensitivity range requested by the World Health Organization for the performance of an antigen test (≥80% sensitivity, ≥97% specificity). If so, dogs could become important allies for the control of the COVID-19 pandemic, especially in developing countries.

Keywords: SARS-CoV-2, COVID-19, sniffing dogs, pathogen detection, dog training, olfactory detection, bio-detection, Mexico

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INTRODUCTION

Since the massive spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) began in late 2019, it has been extremely important to develop fast and reliable detection methods to control the pandemic (1). In many countries, and especially in those with limited economic resources, it has been very difficult to implement mass virus detection programs (2–4). This is because the recommended approaches are high-cost molecular methods such as quantitative polymerase chain reaction (qPCR). Less expensive antigen tests are still non-accessible to developing countries due to their cost as well as difficulty in implementation due to the lack of proper facilities and biosafety systems in place (2, 3). This is a reason why many countries are interested in the use of dogs to detect people infected by SARS-CoV-2. Dogs have more than 220 million olfactory receptors, while humans only have five million (i.e., 2.27% of the number present in dogs) (5). This olfactory potential gives dogs capabilities that can be used to search for people; to detect narcotics and explosives; and to detect diseases such as cancer, malaria, and epilepsy (6–11). Based on these capabilities, it has been inferred that these dogs could help to detect infectious diseases because previous studies have concluded that volatile organic compounds (VOC) produced by infected organisms can be detected by canine olfactory organs (7, 12, 13). In this context, dogs trained to detect SARS-CoV-2 appear as a very promising and cost-effective alternative, as there is evidence of their ability to detect and differentiate VOCs produced by people with COVID-19 (3, 14, 15).

Inspired by the previous work mentioned above, we developed a methodology to train dogs to detect people with COVID-19. In November 2020, we were still training the dogs when the second wave of SARS-CoV-2 infection arrived at our city (Hermosillo, Sonora). At that time, the local government asked us to take the dogs into the field for detection. For this reason, we were allowed to obtain the samples of infected and non-infected people at the Anticipa Health Center in Hermosillo, which makes the present study one of the few attempts to test the performance of COVID-19-sniffing dogs in real life (as opposed to in an experimental setting). However, to get the samples we had to follow the rules of the health providers, who requested that we interact with their patients for the shortest possible time. Thus, we were allowed to collect our three samples; body sweat, axillary sweat, and saliva samples, during only 5 min per patient, which then produced a low level of impregnation of the provided pieces of gauze. We thereby anticipated some decrease in the performance of the dogs but considered it important to share the present results to show the potential of COVID-19-sniffing dogs for middle-income countries like Mexico even under these challenging circumstances.

Our original hypothesis was that our training procedure enables the dogs to detect people with COVID-19. To test this hypothesis, our objective was to determine the sensitivity and specificity of COVID-19-sniffing dogs in field conditions and to compare their performance with the results obtained from the reverse transcription polymerase chain reaction (RT-PCR) and antigen tests for the same patients.

MATERIALS AND METHODS

Sampling Sites

We used sweat and saliva samples of COVID-19-positive and COVID-19-negative humans for dog detection. A convenience sampling methodology was used, obtaining samples from a small number of places during the training and the field study. These institutions were chosen because of their willingness to provide samples. All the samples were obtained from Hermosillo, Sonora, or nearby localities. Specifically, the samples were obtained from the Anticipa Health Center (Hermosillo) (1,932 samples), the General Hospital of the State of Sonora (Hermosillo) (eight samples), Centro Anticipa (Empalme) (eight samples), the Myco textile factory (Empalme) (48 samples), and the University of Sonora (Hermosillo) (eight samples). These samples were used to train the dogs. All the samples were obtained in Sonora between September 8, 2020, and March 31, 2021.

Procedures for Obtaining Sweat and Saliva Samples From COVID-19-Positive and COVID-19-Negative Patients

Before sampling, each patient was asked for her/his willingness to participate in the research, and in the case of a positive answer, a briefing on the objectives of the project and the subsequent use of the samples and epidemiological information was provided. The patient was then provided an informed consent form to read and sign, accepting her/his participation in the project (**Supplementary Material 1**). At the same time, a questionnaire was used to obtain epidemiological data on symptoms and medical history of the patient. The questionnaire collected the following information: full name; age; sex; diagnosed chronic diseases; alcohol, cigarette, or drug use; headache; diarrhea; fever; loss of taste; loss of smell; cough; runny nose; sore throat; body ache; chest pain; nausea; days with symptoms; treatment and days on medication (if provided); and contact with confirmed COVID-19-positive people.

The positive or negative status of each patient was confirmed by RT-PCR performed by the State Laboratory of Public Health and/or by the Panbio COVID-19 Ag Rapid Test Device (Abbott®) antigen test, and in some cases by both tests (see **Supplementary Material 2**). All positive samples came from symptomatic patients or individuals with mild symptoms such as a cold, fever, headache, and/or diarrhea. These data were registered in a database that can be requested from Juan Mancilla-Tapia. The selected samples were from patients who had had symptoms for $\leq$9 days, with a preference for early stage infected who had had mild symptoms for 1–3 days. We also recorded the medications used by each patient (paracetamol, ibuprofen, or other cold medications). Thus, the inclusion criteria for COVID-19-positive patients were (1) age range between 18 and 60 years old, (2) $\leq$9 days of symptoms, and (3) positive for SARS-CoV-2 confirmed by RT-PCR or by the Panbio COVID-19 Ag Rapid Test Device antigen test.

Most of the negative samples were from mildly symptomatic patients, with symptoms like diarrhea, headache, fever, and/or a cold. However, the inclusion criteria for the COVID-19-negative sample collection did not include the most specific

symptoms of COVID-19 such as loss of smell or taste or respiratory problems. Negative samples were also obtained from asymptomatic patients. All negative samples had a negative RT-PCR or Panbio COVID-19 Ag Rapid Test Device antigen test result.

The samples for RT-PCR and the antigen test were obtained from the throat and nasopharynx of all patients following standard procedures recommended by the Mexican health authority (16). The RT-PCR tests were performed by the Laboratory of Molecular Biology of the University of Sonora following the instructions and using the kits recommended by the Mexican health authorities (17). Adequate personal protective equipment was used while collecting the throat and nasopharynx samples and performing the antigen tests. The antigen tests were performed following the instructions of the device manufacturer. All sweat samples, regardless of whether the tests indicated they were positive or negative for SARS-CoV-2, were handled by following the safety measures recommended by the Mexican health authorities (16). Thus, we used KN 95 face masks, nitrile gloves, and all the personal protective equipment recommended by Mexican health authorities. During sample collection, the biological risk was considered "greater than the minimum" because the patients took their own samples under our technical supervision. Technicians did not take samples from patients. This process was approved by the Bioethics and Safety Committee of the University of Sonora. The Mexican health authorities requested that the whole process of providing the briefing, obtaining the consent, applying the epidemiological questionnaire and obtaining the sweat and saliva samples should take no more than 5 min per patient. To collect the sweat samples, each patient was given a pair of non-sterile, dust-free nitrile gloves and a resealable Ziploc® bag containing two 5 cm high, 8 cm diameter translucent glass flasks with metallic caps that had been sterilized in an autoclave and under UVC light, six pieces of new, sterile Jaloma® odorless gauze (10 cm × 10 cm), and four sterile dental swabs. The patient was asked to rub her/his neck, face, and forearms for 1 min with two pieces of gauze on the left half of her/his head, and then wipe the other two pieces of gauze on the right half of her/his head. Subsequently, the patient was asked to place two dental swabs in her/his mouth and one dental swab under each armpit for 1 min. After this time, the patient was instructed to insert the pieces of gauze and swabs with sweat samples into the glass flask, to close it, and to place it back in the resealable bag. Briefly, to collect the saliva sample, each patient was given a pair of non-sterile, dust-free nitrile gloves and a resealable Ziploc® bag containing two 5 cm high, 8 cm diameter translucent glass flasks with metallic caps sterilized in an autoclave and under UVC light, plus two sterile dental swabs. The patient was asked to place two dental swabs in her/his mouth for 1 min. Next, the patient was instructed to insert the dental swabs into the glass flask, to close it, and to place it back in the resealable bag. Again, this process was short, as it took <3 min per patient. No fixatives were added to the saliva samples for training of the dogs. The samples were then transported in coolers to the laboratory and kept there at 18°C until they were used. In the case of the sweat and saliva samples for the field study, the procedure was the same as described above, but the samples were transported immediately to the second floor of the Anticipa Health Center where the dogs and line-up were allocated for COVID-19 detection.

Selection of Canines for Training and Target Odor

Nine dogs were originally selected for training. Dogs without previous training are known as "green dogs" because they have the instinct and performance to pass the different training phases, but they have never been exposed to odors that would affect detection of COVID-19. Six of the nine dogs successfully completed their training. They were a 1-year-old female puppy who was originally in the training process to be an epilepsy detection canine (Leia), two 2-year-old German Shepherd males (Mike and Sam) with no prior training (green dogs from narcotic line), a 1-year-old Belgian Malinois (Krilling) with no prior training (a green dog from narcotic and sport line), and two 2-year-old Belgian Malinois (Harry and Spaidy) without prior training (green dogs from narcotic and sport line). The rest of the dogs did not complete their training for various reasons, such as sickness (not COVID-19) or inability to perform as sniffing dogs. For these reasons, they were allocated to different places as companion pets and are not considered further in this study.

Jendrny et al. (3), Grandjean et al. (14), and Essler et al. (15) have successfully proved that dogs can discriminate between COVID-19-positive and COVID-19-negative individuals based on exposure to different body metabolites excreted through the breath, urine, tears, saliva, feces, and sweat. Grandjean et al. (14) specifically suggested that dogs can detect VOC produced by humans and excreted through their sweat. We concur that dogs have the capacity to detect VOC in human sweat. Our goal was to determine whether this capacity is high enough to be used as a preliminary but effective test to discriminate COVID-19-positive and COVID-19-negative individuals. Thus, we assumed that the odor the dogs are detecting are the metabolites produced by infected humans, and that the dogs are able to discriminate this odor from potentially confounding odors such as those produced by deodorants and anti-transpirants, among many others.

Training Dogs for Odor Detection From Sweat and Saliva

The canine training to detect SARS-CoV-2 lasted ∼12 weeks in the laboratory, with two sessions per day, 5 days a week (Monday to Friday) and one session on Saturday morning. These dogs were the first generation to be trained for COVID-19 detection and were trained exclusively on corporal (as opposed to axillary) sweat samples. All the experiments were performed in air-conditioned experimental facilities at the Obi-K19 canine training center in Hermosillo, Sonora, because the environmental temperature (25–36°C) and low humidity of Hermosillo (https://es.weatherspark.com/m/2272/10/Tiempo-promedio-en-octubre-en-Hermosillo-México) were expected to affect the dogs' performance. Similar negative influences in the performance of dogs have been reported elsewhere (18). Our objective was, that at the end of the training process, the dogs would be able to discriminate the odor (VOC)

of the sweat or saliva of a person with COVID-19 from the sweat or saliva of an uninfected person. The dog training plan had three phases: target odor association, discrimination, and training evaluation.

The target odor association training lasted 3 weeks. Before exposing the dog to the odor produced by people with COVID-19, the dogs were first taught how to use their sense of smell in a targeted manner. Thus, the dogs were trained to mark a toy-type odor. This process consisted of the dog learning two main aspects: the association of marking the toy-type odor with something positive, using the toy as a reward, and training the dog to look for a piece of the toy placed in a stainless-steel box. The toy used for training the dogs is known as the Kong®, and each time they found it in the box, they were allowed to play with it for 3–5 min as a positive reinforcer of behavior. It is important to emphasize that stainless steel was used in all these materials because they do not keep odors. Once the dogs had learned to mark the box with the Kong, they were moved on to the next phase of training.

Once the dogs associated the act of finding something among several boxes with a reward, the objective of reinforcing the desired behavior was achieved. The duration of this phase was 2 weeks. In this phase, a sweat sample was used for the first time. The procedure consisted of allocating a gauze of a COVID-19-positive patient into a stainless-steel salt shaker alongside a piece of Kong because it was an odor the dogs already knew. The purpose of this exercise was for the dog to become familiar with the odor of a sweat sample from a COVID-19-positive patient and to associate it with the reward. This phase of training lasted 4 days. The last part of this phase was the removal of the piece of Kong to leave only the gauze and determine whether the canine marked it correctly. This second step lasted about 10 days. Once the gauze had been used, it was discarded as hazardous biological infectious waste (HBIW). It is worth mentioning that we discovered that the gauze we were using (Protect®, Mexico City, Mexico) had an odor added during the production process. For this reason, we decided to change the gauze, and thereafter we used only a completely odorless gauze by Jaloma® (Guadalajara, Mexico).

In the discrimination phase, we worked with the dogs Harry, Sam, and Mike for 9 weeks and Leia for 11 weeks. The odor of the positive sample was increased by including two or three pieces of gauze for 2 weeks. After that, the discrimination of negative samples began. Two salt shakers were used, one containing sweat swabs from RT-PCR-positive patients and one containing sweat swabs from RT-PCR-negative patients. These samples were placed randomly by a trained assistants in a stainless-steel line with four holes, so neither the trainer nor the researcher knew where the positive samples were. In a subsequent exercise, two salt shakers with negative samples and one with a positive sample were placed, allocating the positive sample in the first hole for the first and second checkups. The positive sample was then moved to the second and third hole in subsequent check-up opportunities. This approach was followed to train the dogs to follow a search sequence. Finally, three salt shakers with negative samples and one with a positive sample were introduced. At this stage, the positive sample was introduced into the first hole during the first and second checkups, moving it to positions two, three, and four in subsequent checkups to reinforce the search sequence to the dog. Subsequently, the dogs were presented with three salt shakers with two negative pieces of gauze (from the same patient) each and a salt shaker with five positive pieces of gauze (from the same patient). The number of positive pieces of gauze was decreased until the dog marked and discriminated samples with the same number of positive and negative pieces of gauze in the salt shakers.

During the training evaluation, we tested the ability of the dogs to distinguish between COVID-19-positive and COVID-19-negative samples. This evaluation was double blind and lasted 2 weeks. Each salt shaker contained the same number of gauze pieces. We used an odor line-up with four holes, presenting one positive sample and three negative samples sequentially for each trial. The dogs were allowed to make a first check of all the samples, followed by a second check when the trainer asked the dog to look for the positive sample. The task of this phase was for the dog to mark five positive samples correctly and consecutively. Once the dog was able to do this, she/he was considered trained. After completing these phases, the dogs entered field work.

A second generation of dogs (Spaidy, Krilling, and Leia) was trained exclusively with saliva of SARS-CoV-2-positive samples from patients with loss of taste and smell. These samples came from the COVID-19 wards of the General Hospital of the State of Sonora and the Cima Hospital of the city of Hermosillo. The procedure to obtain the samples was similar to the one used for sweat samples, with some slight differences.

The second-generation dog training methodology changed slightly. First, instead of the Kong toy, we used food to induce the association of the dog with the saliva sample. Second, we did not include food in the salt shaker together with the COVID-19-positive saliva sample. During the scent association phase, the dogs were only fed on a table that had a flask containing the positive sample. This helped them associate the odor of the COVID-19-positive saliva sample with a reward, in this case food. A second empty vial was then added, and the dog only ate when she/he placed her/his nose in the salt shaker with the positive sample. However, after using food for 3 weeks we decided to go back to the Kong as a reward with two of the dogs (Spaidy and Krilling) because they were originally trained with the toy and performed better than those trained with food. The use of saliva samples reduced the training time to 10 weeks, and after this time the dogs were ready to enter field work. These dogs learned to detect the odor faster than the previous generation due to exposure to samples from positive patients with many of the typical symptoms of COVID-19.

Trial Procedure in Field Conditions and Statistical Analysis

All the samples (positive and negative) for the trials were obtained at the Anticipa Health Center. This Health Center is a two-level building where the samples from patients were obtained on the ground level and immediately taken upstairs to be allocated in the line-ups. For each trial, we used two identical odor lines. Each stainless-steel odor line had four holes, but due to

the shortage of samples, we used only one salt shaker containing a positive sample and one salt shaker with a negative sample. The other two holes in the line had empty salt shakers and were not considered in the statistical calculations. The positive or negative status of the patients to SARS-CoV-2 was based on the Ag test result in 52% of the cases. The positive or negative status of the remaining 48% of the samples was based on symptoms related to COVID-19 and corroborated by the RT-PCR 2 weeks later. Each dog was passed through all the samples in a line a first time for recognition, and after that she/he was passed a second time for definitive detection in the same line. The same procedure was repeated for the second line. In this way, the line-up used in the present study was like that the one described by Kaesler et al. (19). This method apparently improves discriminability by imposing additional memory demand on each dog: They must encode information and the line-up position for the second round. Thus, we slightly modified the methodology proposed by Grandjean et al. (14) as follows: For each trial in a line-up, the dog sniffed each of the two salt shakers for a first time. In the second round, the dog handler asked the dog to look for the positive sample, and the salt shaker the dog marked was considered the definitive identification decision for that trial. For each trial, in contrast to the previous training phases, a sample here comprised two sterile pieces of gauze exposed for 1 min to axillary sweat and one piece of gauze exposed for 1 min to corporal sweat of a patient. These gauzes were introduced in a plastic jar which was sealed for 1–5 min while the technician was setting up the samples. Maintaining the plastic jar sealed for a couple of minutes allowed the odor to impregnate better into the sample. These plastic jars with the sample were allocated in an autoclaved stainless-steel saltshaker 10 cm tall and 7 cm in diameter. The negative sample for that trial was obtained from people present in the health center where the positive samples were obtained and processed in the same way as the positive ones. For each trial, new fresh positive and negative samples were always used, and none of the previous samples were used again. The positive and negative samples were allocated randomly by the data recorder, and neither the dog handler nor the dog knew where the positive samples were. In fact, both the dog handler and the dog were looking in a different direction when the salt shakers were allocated in the line-up (double-blind strategy). The recorder indicated that the line-up was ready and it was at this moment that both the dog handler and the dog faced the line-up. Once the dog sniffed all the salt shakers and marked one (by sitting, or laying on it) the dog handler made a signal (upright closed fist) to indicate that the trial had finished. The data recorder indicated verbally whether the mark was correct, and if so, the dog handler immediately rewarded the dog with the Kong, allowing him/her 2–3 min to play with the toy. The exposure procedure of the dogs to saliva samples was the same as for sweat samples, with the only exception that each plastic jar with two dental swabs positive for SARS-CoV-2 was allocated and opened in a sterilized salt shaker. The negative samples were two clean dental swabs in a plastic jar that was introduced and opened in a sterilized salt shaker. The testing period lasted for 12 weeks, during which time none of the dogs showed disease signs.

Sensitivity and specificity were calculated as recommended by Johnen et al. (20) because the line-up was a mixture of simultaneous and sequential line-up as explained above. Each trial was a typical Bernoulli experiment where the probability of success or failure is 50% (like the flip of a coin). Thus, after many Bernoulli experiments exposing the dogs to COVID-19-positive and COVID-19-negative samples, we were able to calculate 95% confidence intervals (CI). If a 95% CI does not overlap 50%, which is the randomness region, that 95% CI could be considered significantly different from a random choice. Thus, we calculated 95% CI of the sensitivity and specificity with Clopper–Pearson's method, using the package epiR (https://cran.r-project.org/web/packages/epiR/index.html), and considered significant those 95% CI that did not overlap $\leq$ 50% sensitivity and specificity values. We followed the procedures recommended by Trevethan (21) to calculate sensitivity and specificity. The minimum number of samples to be sniffed for an adequate study power was calculated assuming a 15% of prevalence of SARS-CoV-2 in the population. This was a rather conservative estimation of prevalence since by November 2020, we were in the middle of the second wave of SARS-CoV-2 at Hermosillo. The probability of type 1 error, determining that there is a difference when such difference does not actually exist, was established at 0.05. The power of the analysis to detect a difference between groups when such a difference exist was assumed to be 80%. All the calculations for the number of samples to be sniffed were made with ClinCalc.com (https://clincalc.com/stats/samplesize.aspx). These calculations gave us a sample size of 94 to have an adequate study power.

RESULTS

The sweat and saliva samples were obtained from a total of 138 people at the Anticipa Health Center, Hermosillo, Sonora (**Supplementary Material 2**). Of the 138 samples of sweat, 69 were positive to SARS-CoV-2, and 69 were negative. In the case of saliva, 128 samples were obtained, from which 54 were positive to the virus and 74 were negative. The whole sample comprised 59% women and 41% men. The group of positive people comprised 59% women and 41% men, and the group of negative people was 42% women and 58% men. There were

TABLE 1 | Details of the six fully trained canine participants exposed to sweat and saliva samples of COVID-19-positive and COVID-19-negative humans.

Name	Sex	Age (years)	Breed	Specialty
Sam	Male	2	German Shepherd	Green dog*
Leia	Female	1	Golden retriever	Epilepsy
Mike	Male	2	German Shepherd	Green dog
Harry	Male	2	Belgian Malinois	Green dog
Krilling	Male	1	Belgian Malinois	Green dog
Spaidy	Male	2	Belgian Malinois	Green dog

**A green dog is one that had not been trained previously to detect any kind of odor.*

no differences between the COVID-19-positive and COVID-19-negative groups in the proportion of women (Fisher's exact test, difference between proportions = −0.02, p = 1) and men (Fisher's exact test, difference between proportions = −0.008, $p = 1$). The age range of the whole sample was between 18 and 60 years, with 37 ± 10 years for women and 38 ± 12 years for men. The age in the positive group was 39 ± 11 years for women and 37 ± 18 years for men; there was not a significant difference in age between the sexes (Student's $t_{0.05} = -0.49$, $p = 0.62$). The age in the negative group was 35 ± 9 years for women and 39 ± 11 years for men; there was not a significant difference in age between the sexes (Student's $t_{0.05} = 1.27$, p = 0.21). There were no differences in the mean age between the COVID-19-positive and COVID-19-negative groups (Student's $t_{0.05} = -0.07$, $p = 0.94$). The characteristics of the dogs exposed to sweat and saliva of COVID-19-positive and COVID-19-negative patients are presented in **Table 1**.

Table 2 shows the sensitivity and specificity for the four dogs exposed to sweat samples compared with the results of RT-PCR and the antigen test. Sam and Leia had a marginal performance and their 95% CI overlapped the randomness region (50%). In contrast, Mike and Harry had sensitivity values of 76 and 80%, respectively, and their 95% CI were far from the randomness region. The specificity of Sam and Leia also overlapped the randomness region. In contrast, the specificity values for Mike and Harry were 75 and 88%, respectively, and their 95% CI did not overlap the randomness region.

Table 3 shows the sensitivity and specificity for the three dogs exposed to saliva samples compared with the results of RT-PCR and the antigen test. The dogs had sensitivity values between 70 and 78%, and for Spaidy and Krilling, their 95% CI did not overlap the randomness region. In contrast, the 95% CI of Leia overlapped the randomness region. The specificity for the three dogs followed a similar pattern, where the 95% CI for Spaidy and Krilling did not overlap the randomness region, while the one for Leia did.

DISCUSSION

We originally hypothesized that our training procedure enables dogs to detect people with COVID-19 with a sensitivity and specificity significantly different from random. In that sense, all the dogs, with the exception of Sam and Leia for sweat (**Table 2**) and Leia for saliva (**Table 3**), had sensitivities significantly different from random. This was evident from the 95% CI for Mike and Harry (**Table 2**) and for Spaidy and Krilling (**Table 3**). Thus, we partially proved our hypothesis as correct. Moreover, the performance of the dogs was outstanding in view of the challenging circumstance in the field study: the pieces of gauze were exposed for no more than 5 min to axillary sweat or saliva. However, it is also evident from **Table 2** that with the exception of Harry's 95% CI, none of the remaining dogs reached a sensitivity of ≥80%. It is desirable to reach or surpass this value to meet the World Health Organization (WHO) requirement for validation of antigen tests (22). If this sensitivity level is reached, then COVID-19-sniffing dogs could be considered at least as sensitive as the antigen tests currently available. In the case of specificity, the results for the dogs were not good: None of our dogs reached the ≥97% threshold established by the WHO for validation of antigen tests (22). Two mutually exclusive explanations for this result that together with other variables that could have affected the dogs' performance are presented below.

Our results suggested that the age range and sex of the people participating in the study were not relevant for the performance of the dogs. Although the age range was wide (18–60 years), the mean ages of women and men were very

TABLE 2 | Sensitivity and specificity of the four dogs trained to detect COVID-19 from the sweat of positive and negative people compared with the reference tests (Antigen test and RT-PCR).

Name	*n*	Sensitivity (%)	95% CI	Specificity (%)	95% CI	PPV (%)	95% CI	NPV (%)	95% CI
Sam	132	58	45–71	69	57–80	61	48–74	67	55–77
Leia	132	60	47–72	64	52–76	62	49–74	62	50–74
Mike	124	76	63–86	75	63–85	74	61–84	78	66–87
Harry	95	80	66–91	88	75–95	86	72–95	83	70–92

CI, confidence interval; n, number of trials; NPV, negative predictive value; PPV, positive predictive value; RT-PCR, reverse transcription polymerase chain reaction.

TABLE 3 | Sensitivity and specificity of the three dogs trained to detect COVID-19 from the saliva of positive and negative people compared with the reference tests (Antigen test and RT-PCR).

Name	*n*	Sensitivity (%)	95% CI	Specificity (%)	95% CI	PPV (%)	95% CI	NPV (%)	95% CI
Spaidy	138	70	56–82	69	58–78	58	45–70	79	68–87
Krilling	138	78	65–89	69	58–78	59	46–71	85	75–92
Leia	32	73	45–92	53	28–77	58	33–80	69	39–91

CI, confidence interval; n, number of trials; NPV, negative predictive value; PPV, positive predictive value; RT-PCR, reverse transcription polymerase chain reaction.

similar in COVID-19-positive and COVID-19-negative groups. There were no significant differences between the mean values of age of individuals or the proportion of females and males in the COVID-19-positive and COVID-19-negative groups. In addition, 97% of the people who provided samples came from just one place, the Anticipa Health Center in Hermosillo. Consequently, these similarities suggest that the samples of infected and non-infected people came from the same population and that both groups were comparable. This is important because, as mentioned by Grandjean et al. (14), significant differences in age between the groups being compared (infected vs. non-infected) cast doubts about the influence that this variable could have on the dog's performance to detect COVID-19-infected people, because body smell changes with age (23, 24). With respect to gender, we did not find significant differences between infected and non-infected individuals; hence, we met the comparability criterion for detection dog studies recommended by Edwards et al. (25).

Because most of our dogs did not overlap the randomness region (50%), we considered that the procedure for training them to detect people with COVID-19 was successful. The training procedure of the first generation of dogs lasted 4.5 months and was based on dog training procedures for the detection of narcotics, explosives, and epilepsy. The results showed that in 4.5 months, 50% of the dogs were able to reach sensitivity levels between 75 and 83%, and the other 50% reached sensitivity levels between 54 and 67% in the laboratory. Here, it is important to mention that with the exception of Leia, all other dogs were "green dogs" from narcotic line without previous experience in detection. Moreover, this is the first generation of dogs trained to detect COVID-19 in Mexico and, clearly, we dealt with a learning curve. This is especially noticeable when we compare our results with those of more experienced research groups who have reported a training time for detection of COVID-19 between 1.75 (26) and 3.75 months (27). This is certainly a matter of experience because a second generation of dogs has been trained in 2 months and are almost ready for their first field experience.

Based on the experience acquired by other groups working with dogs for COVID-19 detection and our own experience, we suggest that the training of dogs can be improved and the training periods shortened by: (1) using pseudo-scents (training aids in which the true material—SARS-CoV-2 in this case—is not part of the compound) (28), but only some of the chemical compounds produced by the human in response to the virus. These types of aids are commonly used during dog training for detection of bombs, narcotics, or human cadavers (29). In the specific case of COVID-19 sniffing dogs, there is a need of further scientific evidence on whether these aids increase the detection capacity of the dogs. However, pseudo-scents are considered as very useful for training dogs for the detection of narcotics such as cocaine (30). (2) The use of trained dogs which apparently could increase the speed at which they become proficient in the detection of COVID-19 to a few weeks compared with several months to over a year for juvenile green dogs without detection training. The previous point seems to be critical, but whether dogs belong to pure or mixed dog breeds seems to be irrelevant (14, 27, 31), and (3) exposing the dogs to samples of different respiratory tract infections together with SARS-CoV-2 samples during their training period as ten Haggen et al. (13) have recently demonstrated.

The present study is one of the first field experiences exposing trained dogs to sweat and saliva samples immediately after they have been acquired from patients. Unfortunately, the short time we were allowed by the health center to expose the pieces of gauze to the armpit and mouths of the patients probably negatively affected the performance of the dogs (**Tables 2**, **3**). Thus, our results suggest the need to keep the pieces of gauze in the patients' armpits or mouths for at least 10 min, as recommended by Grandjean et al. (14), and ideally 20 min as other authors have done (32). Even under these challenging circumstances, the dogs performed relatively well. In fact, one of our sweat-sniffing dogs (Harry) reached 80% sensitivity, with a 95% CI that was far from the randomness region. This is considered a good result because it falls within the range requested by the WHO for the performance of the antigen test (22). Mike, another sweat-sniffing dog, was very near this sensitivity value (76% sensitivity; **Table 2**) with his 95% CI overlapping the 80% sensitivity value. The other two sweat-sniffing dogs did not reach 80% sensitivity with their 95% CI. Clearly, these were the dogs most affected by the short exposure time of the pieces of gauze to the patients' sweat. In the case of saliva (**Table 3**), neither Spaidy nor Krilling reached 80% sensitivity. However, the 95% CI of both saliva dogs overlapped this sensitivity value. Thus, it is highly probable that if the exposure time increased, the dogs would have a better performance. In the case of Leia, her sensitivity to saliva samples reached 73%. However, her 95% CI was highly variable (0.45–0.92), overlapping the randomness region. This was probably due to the low number of trials (32) to which she was exposed. Clearly, with more trials, the 95% CI values become more reliable as in the case of all other dogs in **Tables 2**, **3**. Thus, the experience acquired in this case suggest that the number of trials should be at least 100, but it would be much better well beyond 100 trials to reach a reliable estimation. Note that this number is similar to the one obtained by the study power analysis ($n = 94$).

With respect to specificity, the results in **Tables 2**, **3** suggest that the dogs were affected negatively by the short time we were allowed by the health center to expose the pieces of gauze to the patients' armpits and mouths. The most likely explanation for this low level of specificity is that the dogs are not identifying COVID-19 only, but also other respiratory infections. All the people who participated in the present study were there because they were experiencing symptoms of an infection of the respiratory tract and looking for a SARS-CoV-2 test. It is likely that many of these people were infected with respiratory infections other than COVID-19 as shown by the negative RT-PCR and antigen test results. It may be prudent to consider that during their training, the dogs should be exposed to other respiratory infections at the same time they are exposed to SARS-CoV-2 as ten Haggen et al. (13) have recently suggested. Thus, it is highly possible that the dogs were identifying chemical clues related to a general response of the patients to those infections (including COVID-19), as D'Aniello et al. (33) proposed. These authors pointed out that all previous experiences published

have been experimental exposures of the dogs to sweat, saliva, or tracheobronchial secretions of healthy or sick people with COVID-19. Thus, they emphasized the need to expose the dogs to real-life conditions to determine their performance, and they predicted that a decrease in the performance of the dogs could be expected due to the many odors present in hospitals or health centers. We attributed the low level of specificity reached by our dogs to the confounding variables generated by different infections of the respiratory tract in the people present in the Anticipa Health Center. Grandjean et al. (32) have also suggested that several health conditions of the positive patients could act as confounding factors for the dogs' performance to detect COVID-19. We also concur with Essler et al. (15) and D'Aniello et al. (33) on the need for a careful study of VOC produced by the people with COVID-19 and non-infected people. However, a further concern that should be raised is the need for specific identification of the respiratory infections of the false-positive people marked by the dogs. This information would allow matching the identity of the infection with the profile of VOC produced by the patients to better tune the dogs' training.

In addition to the low specificity of the dogs due to the possible detection of other respiratory infections, there are other confounding factors that should be addressed in future work. Apparently, working with the dogs every day 5 days a week with two sessions per day negatively affects the dog's performance. Thus, an additional potential explanation for the poor performance of our dogs could be related to this fact. In contrast, Mendel et al. (34) obtained high positive predictive values (73.7–93.9) exposing the dogs three times per week to COVID-19 samples. This point deserves careful consideration because it certainly increases the number of dogs needed for detection. Another problem to solve is the immediate access to the COVID-19 test result of the person being sniffed to know whether the dog should be rewarded. In fact, in our study the RT-PCR results were delayed from 2 days up to 2 weeks. We solved this problem partially by asking the people for a sample for an antigen test. However, there are two additional problems with this procedure. First, antigen tests are not as sensitive as the RT-PCR test, especially if the person has an early infection and is still building up the viral load. Second, the people are reluctant to provide another nasopharyngeal sample because it is painful or at least uncomfortable, especially after the sample extraction for the RT-PCR. An alternative is to use DNA/RNA shield saliva/sputum collection kits to collect samples of symptomatic and asymptomatic people, combined with rapid molecular tests such as RT-LAMP. These seem to be easy ways to obtain reliable samples to detect the virus (3, 34).

An advantage of the use of dogs is their capacity to detect infected people before RT-PCR or the antigen test. If a patient had loss of taste and/or smell and negative RT-PCR and antigen test, but the dogs alerted the sample was positive, we decided to follow up with the patient by telephone, allowing us to know their health status and, if a second test was carried out, to know its result. Due to the follow-up of the patients, six cases were detected that originally had a negative RT-PCR and the dogs marked the patient as positive. Two to five days later, the patients who were followed up underwent a second lab test. Two of those cases were checked with the antigen test, three had RT-PCR, and one case had an antibody test. The results of these six follow-up cases were positive for SARS-CoV-2, agreeing with the results of the dogs. Similar results have been reported by Grandjean et al. (14) and Carvalho et al. (35), who found two people and one person, respectively, who the dogs marked as positive, but their RT-PCR tests were negative, and a few days later they had the test again and were positive. Thus, our results and those of the researchers mentioned above suggest dogs' performances are likely better than reported because of the somewhat flawed RT-PCR reference standard test used for comparison. Further, the antigen tests are much less accurate than the RT-PCR test, so dog "errors" based on these tests are even more unreliable and can lead to poorer dog sniffing results [see (36) for an excellent comparison of the performance of RT-PCR and antigen tests for detection of SARS-CoV-2]. Certainly, this is an area that deserves careful attention.

One of the main ethical concerns regarding the use of sniffing dogs is the potential risk for them to become infected due to exposure to COVID-19-positive samples. To try to diminish this risk, we used stainless-steel salt shakers that act as physical barrier to avoid compromising the dogs' health. Fathizadeh et al. (37) showed that sweat of the hands of people with COVID-19 did not contain the virus. However, there is no information published on the presence of the virus in sweat of other human body parts. Therefore, the use of salt shakers appears to be a reasonable option for the dogs to sniff only VOC without physical contact with the gauze impregnated with the sweat or saliva of infected patients. Other methods of inactivation of the samples of SARS-CoV-2 such as beta-propiolactone (3), NP-40 detergent and heat (15) have been used. In addition, it has been demonstrated that the use of UV radiation to inactivate SARS-CoV-2 does not change the VOC composition of face masks of infected people exposed to dogs (34). Thus, apparently, these preventive sample treatments do not affect the quality of the samples at all. The use of these methods to prevent a dog's infection is crucial since they can become infected with the virus, even when the published evidence suggests that their infection level can be from low (38, 39) to no infection at all (40).

In conclusion, due to the challenging conditions of only 3 min exposure of the pieces of gauze to axillary sweat and saliva, only one of our dogs reached 80% sensitivity. However, another sweat-sniffing dog (Mike) and two saliva-sniffing dogs (Spaidy and Krilling) had 95% CI that did not overlap the random region (50% of sensitivity). These results seem promising even though only one dog reached the sensitivity range requested by the WHO for the performance of an antigen test (22). In the case of specificity, the results were not favorable, but apparently the dogs are able to detect people sick not only with COVID-19, but also other kind of respiratory diseases. We are almost certain that if the pieces of gauze were left under the armpit or the buccal swabs were left in the mouth for at least 10 min, the dogs would show improved sensitivity and specificity. This eventuality is very important because if the dogs reach the sensitivity and specificity ranges required by the WHO for the performance of antigen tests, they could become a crucial ally alongside other molecular and antigen test to control the COVID-19 pandemic. This claim is

based on the fact that if we are able to detect COVID-19 in a fast and reliable way, we could isolate the infected individuals and thus decrease transmission dramatically. Very strong support for this view and for the role of COVID-19-sniffing dogs is the mathematical modeling simulations of Larremore et al. (41). These authors suggested that effective screening of COVID-19 depends largely on the frequency of testing and the speed of reporting rather than high test sensitivity. If this is so, then COVID-19-sniffing dogs have a bright future, because even if their sensitivity and specificity levels decrease in the field, they still can provide very fast and reliable results each day for several years.

DATA AVAILABILITY STATEMENT

Datasets are available on request. The raw data supporting the conclusions of this article will be made available by JM-T (jmancilla@obi-k19.com) without undue reservation.

ETHICS STATEMENT

The studies involving human participants and animal study were reviewed and approved by Comité de Ética en Investigación de la Universidad de Sonora. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JM-T and VV-M conceptualized the study, drafted the manuscript, tables, and prepared the final version. VL-E, AO, RO-C, RR-Z, BM-C, JB-C, IR-L, CG-B, and AN-G collected and compiled data. All authors contributed to data interpretation, critical revision, and approved the final manuscript for submission.

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REVIEW
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The Use and Potential of Biomedical Detection Dogs During a Disease Outbreak

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Biomedical detection dogs offer incredible advantages during disease outbreaks that are presently unmatched by current technologies, however, dogs still face hurdles of implementation due to lack of inter-governmental cooperation and acceptance by the public health community. Here, we refine the definition of a biomedical detection dog, discuss the potential applications, capabilities, and limitations of biomedical detection dogs in disease outbreak scenarios, and the safety measures that must be considered before and during deployment. Finally, we provide recommendations on how to address and overcome the barriers to acceptance of biomedical detection dogs through a dedicated research and development investment in olfactory sciences.

Keywords: biomedical detection dog (BMDD), canine, olfactory science, training aid delivery device (TADD), COVID-19, volatile organic compound (VOC), volatilome

INTRODUCTION

Detection dogs have played a role in society since the Middle Ages, depicted wearing armor alongside knights and the familiar howl of the bloodhound as it tracks down criminals or missing people. In modern society, detection dogs are most often seen in a law enforcement capacity, screening people, luggage, vehicles, and cargo for contraband. However, a trend is emerging in which dogs' olfactory abilities are being harnessed to not only detect a growing list of contraband, but also in an increasing number of fields and applications completely outside of law enforcement. A small selection of these detection disciplines is listed in **Table 1**.

There are approximately 10,000 law enforcement working dogs in the United States amongst the military, federal, local, and state police agencies (1). These working dogs are present on our military bases, in our transportation hubs (e.g., train stations, airports, seaports), and on the streets of every major city in the United States. Another way of looking at these numbers and their geographical and situational distribution is to see the potential of having a network of highly adaptable sensors all throughout the country, able to detect any threat with a reproducible odor. The current COVID-19 pandemic has shown that globally, we were not prepared to handle an outbreak of that magnitude, especially of an unknown pathogen. Since there was no immediate understanding of the infectivity and transmissibility of the virus, there was a willingness to look outside the typical methods for pathogen detection and identification; potentially repurposing prophylactics, treatments and/or diagnostic/detection equipment. Ultimately, there was a need to investigate our most primitive (but not unsophisticated), yet reliable form of detection, canine olfaction.

TABLE 1 | A selection of examples demonstrating the growing list of detection dog disciplines.

Contraband
○ Explosives
○ Narcotics
○ Firearms
○ Currency
○ Agricultural products
○ Exotic animals or animal products
○ Lithium-Ion Batteries
Live human
○ Search and rescue
○ Patrol/Apprehension
○ Tracking/Trailing
Forensics
○ Human remains/Cadaver (dead humans)
○ Bodily fluids
○ Arson/Accelerant/Fire Inspection
○Human scent
Conservation
○ Endangered/Threatened species
○ Site surveys to assess the effect of infrastructure on animal habitats
Electronics (storage devices, mobile phones)*
Hobby/Sport
Biomedical (**Table 2**)

**Falls into both the forensics and contraband detection categories.*

Much of what is needed to address and terminate an outbreak is pathogen-dependent. Typically, the pathogen must be isolated, identified, cultured, its genetic material sequenced, and only then can the scientific community begin to develop effective vaccinations, therapeutics, and diagnostics. In the meantime, the community follows the "Swiss Cheese" model, relying on personal responsibilities such as personal protective equipment (PPE) (e.g., masks), social distancing, frequent handwashing, and cough etiquette (2) to combat the general spread of germs, but not the detection of the pathogen. But what can be effective while we wait for the scientific community to ramp up, is canine-based detection as canines only rely on the pathogen or the disease-state odor. We do not even need to necessarily have that odor's volatile organic compound (VOC) profile characterized, we just need a way to safely capture/reproduce, store, and present the odor to the detection dogs. This odor detection scenario is obviously a gross oversimplification of the process, but it is currently the most straightforward of all of our detection capabilities. One should note that at this time Biomedical Detection Dog (BMDD) capabilities are considered detection or screening tool and not diagnostic technology. The distinction being that to be a diagnostic, BMDDs would need approval from the United States Food and Drug Administration (FDA) (3).

Beginning with the 1989 (4) and 2001 (5) case reports of patients' pet dogs causing concern due to the excessive sniffing their dogs conducted at suspicious moles that were later determined to be cancerous, the ability of dogs to sniff out disease has grown from anecdotal to a full-fledged scientific discipline. Now BMDDs work as part of research teams in prestigious academic institutions such as the University of Pennsylvania's PennVet Working Dog Center (established 2012), detecting ovarian cancer, sinonasal inverted papilloma, COVID-19, Spotted Lanternfly infestations, biofilms, and chronic wasting disease (6). An established body of literature exists demonstrating the effectiveness of dogs and their ability to detect the VOC signatures associated with disease including, but not limited to, toxigenic *Clostridium difficile* in stool (7), lung and breast cancers in breath (8), four different bacteria causing urinary tract infections in patient urine samples (9), bovine viral diarrheal virus (BVDV) infected cell-cultures (10), supernatant from *Pseudomonas aeruginosa* cultures (11), parasitic *Plasmodium falciparum* (malaria) infection using patient clothing (12), prostate cancer in urine (13), ovarian cancer in blood (14, 15), type 1 diabetes (16), and Parkinson's disease (17) in sebum. Disease detection by canines has been systematically reviewed by Moser and McCulloch (18), Edwards et al. (19), Cambau and Poljak (20), and Salgirli Demirbaş et al. (21) and reported to be a scientifically sound method of detection.

BMDD history can be roughly categorized into three periods of time: the beginning starting with the 1989 case report of melanoma and culminating in 2010 with the Moser et al. review "Canine scent detection of human cancers: A review of methods and accuracy" wherein six published studies on canine detection of human cancers were reviewed in depth. This beginning period focused nearly exclusively on canine detection of cancer. The next period runs approximately from 2010 to 2020 in which the field of biomedical detection dogs expands beyond cancer and into the variety of subdisciplines (**Table 2**). This ten-year period is marked by an explosion of canine detection research resulting in a growing list of detectable human diseases by BMDDs and BMDDs able to detect **virus** [bovine viral diarrhea virus (10)], **bacteria** [C. difficile (7), Escherichia coli, Klebsiella pneumoniae, Enterococcus faecalis, and Staphylococcus aureus (9)], **pests** (brown tree snakes (22), palm weevils (23), gypsy moths (24), longhorn beetles (25), termites (26), bed bugs (27), and quagga and zebra mussels (28), fouling agents [catfight off-flavoring compounds (29), microbial growth in buildings (30)], **animals important to conservation efforts** [grizzly and black bears (31), brown bears (32), geckos and tuataras (33), tortoises (34), quolls (35), jackals (36), giant bullfrogs (37), wolves (38), rabbits (39), rock ptarmigans (40), bats (41), koalas (42), kit foxes (43), tigers (44), cougars (45), cheetahs (46), bobcats (47), and gorillas (48)], and **disease odor directly on humans** [Parkinson's (49), epilepsy (50), diabetes (16, 51)].

The third period of BMDD history began in early 2020, coinciding with the SARS-CoV-2 global pandemic. Research groups from around the world, already deeply knowledgeable about the abilities of canines to detect human diseases, embarked on proof-of-concept studies to determine if BMDDs would be able to detect a human disease caused by a virus, in the midst of a pandemic caused by said virus. Based upon BMDD detection of the wide variety of human diseases and BMDD detection of a virus (BVDV), all of the evidence supported this as a valid next step for canine detection. The novel aspect of what was being attempted was BMDD detection of a human disease (COVID-19) caused by a virus (SARS-CoV-2). The global success

TABLE 2 | Subdisciplines within the biomedical detection dog field and examples of the diseases/pathogens/pests they detect.

Biomedical detection dogs*	
DISCIPLINE	**EXAMPLES**
Medical detection (Detects disease state, i.e., signature volatilome or change in volatilome produced by infected hosts)	Non-infectious: • Cancers: Melanoma (113) • Altered Metabolic Status: Diabetes (16) Infectious: Malaria (12)
Agricultural disease detection	Potato virus Y (PVY), the etiological agent of Potato Tuber Necrotic Ringspot Disease (PTNRD) (114)
Biological detection (Detects pathogen)	Bovine Viral Diarrheal Virus (BVDV) (10)
Pest/Invasive species detection	Pests: Bed bugs Invasive Species: Asian longhorn beetle, *Anoplophora glabripennis* (25)

**While detecting a biological organism, for the purposes of this review, biomedical detection dog (BMDD) specifically does not include conservation, forensic, and live human detection dogs as these detection disciplines would not be directly relevant to disease detection during an outbreak scenario.*

of the COVID-19 detection dogs demonstrated the efficacy of BMDD detection of virus-induced human disease, but more significantly, it demonstrated the potential for BMDDs during a disease outbreak.

Five significant COVID-19 BMDD research highlights over the past 2 years are that these dogs:

(1) were trained, tested, and evaluated at research institutions or utilized in some capacity in at least twenty-five countries [Argentina (52), Austria (53), Australia (54), Belgium (55), Brazil (56), Cambodia (57), Canada (58), Columbia (59), Chile (60), Czech Republic (61), El Salvador (62), Finland (52), France (63), Germany (64–66), India (67), Iran (68), Italy (69), Lebanon (52), Russia (70), South Africa (71), Switzerland (72), Thailand (73), United Arab Emirates (74), United Kingdom (75), United States of America (76)] and, when assessed, demonstrated results in sensitivity and specificity, ranging from 65 to 100% and 76 to 99% (77), respectively, illustrating the consistency and robustness of their detection accuracy despite the differing training methodologies employed,
(2) were deployed in at least four countries (Finland, Lebanon, UAE, and United States) screening people for COVID-19 in airports (78, 79),
(3) demonstrated the ability in one study to achieve detection sensitivities greater than the gold standard real-time polymerase chain reaction (RT-PCR) and in less time (80), demonstrating their potential role in medical diagnostics,
(4) distinguished COVID positive from COVID negative samples with similar efficacy regardless of body fluid sampled (i.e., saliva, urine, and sweat) (66) demonstrating the range of non-invasive samples that BMDDs are capable of utilizing in a pandemic, and
(5) in one study, were able to differentiate SARS-CoV2 infections from infections with other novel coronaviruses, influenza viruses, parainfluenza viruses, an adenovirus, a rhinovirus, a metapneumovirus (HMPV), and respiratory syncytial virus (RSV)—all etiological agents common to respiratory tract infections (65), thus demonstrating the potential for BMDDs to aid in the triage and differential diagnosis process.

Utilizing the COVID-19 BMDDs as an example, one of the first questions to address during a disease outbreak would be if the BMDDs were able to detect the pathogen or disease and to what extent. The sensitivity and specificity of COVID-19 BMDDs has been reviewed in depth (77, 81–84) and the answer to this question is an overwhelming "yes." Now that it has been irrefutably established that BMDD detection of a pandemic human disease caused by a virus is not only possible but that it is faster and more sensitive than our gold standard diagnostics, the question is what is the potential for BMDDs going forward for the next disease outbreak and what are some of the considerations that should be made around BMDD deployment.

What Is a Biomedical Detection Dog?

For the purposes of this review, the term biomedical detection dog (BMDD) is an all-inclusive term to include: medical detection dogs that detect diseases in humans, agricultural disease detection dogs, and biological detection dogs that detect the microorganisms or etiological agents themselves, and to a smaller extent pest/invasive species detection dogs that detect primarily nuisance plant, animal, or insect life or invasive species as defined by Executive Order 13,112 ". . .as an alien species whose introduction does or is likely to cause economic or environmental harm or harm to human health" (**Table 2**).

One should note that these disciplines are not mutually exclusive and certain diseases and canine training approaches could transcend into multiple areas. For example, COVID and SARS-CoV-2 detection dogs, if the dog is trained to detect the disease state produced by the human in response to infection, that would fall into the medical detection dog category, however, if the dog is trained to detect the viral proteins produced during the course of infection, i.e., the etiological agent, then that would fall into the biological detection dog category (see **Figure 1** for example).

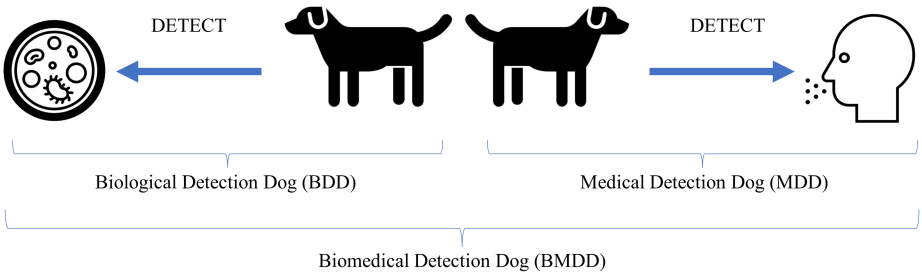

FIGURE 1 | Illustration of biological and medical detection dogs and what they detect. The Biological Detection Dog (BDD) detects the odor of (or odors associated with) the pathogen or etiological agent, while the Medical Detection Dog (MDD) detects the odor of (or odors associated with) the disease state produced by an infected host in response to the pathogen or the altered volatilome due to disease not caused by an infectious agent. Together, BDDs and MDDs can be categorized as Biomedical Detection Dog (BMDDs).

Why the convergence of terms? Because the line between biological and medical is increasingly becoming blurred. COVID-19 brought this to the forefront as there were dogs trained to detect the COVID-19 disease state and dogs trained to detect the pathogen, SARS-CoV-2. Viruses are not living without a host and we are not training the dogs to detect the actual whole virus particles, or the culture media, but the viral proteins are produced by the host, with the odor resulting from both virus (pathogen) and human (host). When we consider if this falls into the biological or medical detection, it seems to fall squarely into biomedical detection. Perhaps until we know more as to what exactly the dog is detecting odor-wise, this broad category is appropriate. Or perhaps increased knowledge into what the dogs are detecting will only perplex us more as to how dogs are able to detect the signal from the noise in these incredibly complex backgrounds.

What Are the Potential Applications of a Biomedical Detection Dog?

In most disease outbreak scenarios, biomedical detection dogs could serve a role in the detection of either the disease state or the pathogen directly. Early in the COVID-19 pandemic, before rapid diagnostics were widely available and once the general public started to become aware of the ability of canines to detect diseases and specifically COVID-19, prominent members of the canine detection community were fielding inquiries from all over the world on the potential applications and abilities of BMDDs. **Table 3** lists many of the locations in which COVID-detection BMDDs either were (since 2020) or could be deployed in a disease outbreak scenario. The most obvious deployment scenarios were in transportation hubs such as airports and railway stations to quickly screen passengers during their travels in an attempt to stop the spread of the virus due to global travel. The majority of the scenarios involved active screening in which BMDDs would be actively searching the traveling public as they move from one location to another; however, other scenarios soon gained interest as institutions and businesses sought ways to not only re-open but to stay open during an ongoing pandemic. These latter scenarios called for BMDDs that monitor a relatively consistent resident population in a given area for changes in their infectious status. Since dogs are able to detect subtle changes in the volatilome (the odor actively being released), and often recognize signs of infection before and more accurately than traditional diagnostics, BMDDs offer a potential early warning system to alert us to the presence of an infected individual before they know they are ill, or before they demonstrate some of the more canonical signs of infection (e.g., fever, chills, aches, nausea, cough) (68, 80).

One of the most compelling use cases for BMDDs during a disease outbreak is in the medical care or hospital setting. BMDDs are capable of screening hundreds of people in a non-invasive manner, a sniff of the airspace around the person, in less than an hour. This capability can be used to triage long lines of people waiting to get tested or enter medical facilities. Instead of using an inefficient and hazardous first-come-first-served approach, the BMDDs can assist in identifying the people who are most likely positive for the disease, isolate them in a separate area, and expedite their tests. Selecting the presumptively positive individuals from the testing line, increases testing efficiency, removes the infection from the zone of susceptible people around them, and shortens that critical time to diagnosis (TTD) window which helps medical personnel take the proper disease precautions and administer the appropriate medical care, and allows faster allocation of limited medical resources (personnel and supplies).

Should a disease outbreak be so severe that PPE and test equipment were again to be in short supply or non-existent, BMDDs could also serve a role in helping triage the use of these items in the decision-making process before patient treatment. In this scenario, it is possible that it would be necessary to rely upon BMDDs to make the preliminary presumptive positive detection so that diagnostic tests are, in theory, only utilized on positive patients and thus the associated PPE, medical supplies, and testing equipment/kits would be prioritized and spared.

Disease outbreaks, epidemics, and pandemics can arise from different sources, either natural or "man-made." Naturally occurring infectious diseases follow the typical chain of infection whereby disease transmission occurs when the pathogen leaves its reservoir and is transmitted to a susceptible host. For example, the disease malaria occurs when a Plasmodium (etiological agent) infected mosquito (reservoir) bites (mode of transmission) a human (susceptible host). Disease outbreaks could also arise

TABLE 3 | Locations where biomedical detection dogs have or could be deployed during a disease outbreak.

Location(s)	Purpose/application
Schools (115)	One-time screening of visitors
Prisons	Periodic screening of traveling public
Work Sites/Buildings (116)	Confirmation of Negative COVID Tests for Entry
Ships (Naval, Cruises, Cargo) (117)	Surveillance screening of resident population (e.g., assisted living residents)
Assisted Living Facilities (116)	Daily screening of personnel (e.g., workers, teachers, students)
Farms*	Patient triage
Transportation Hubs (Airports, Railways) (78)	Sample screening
Border Crossings	
Hospitals (58, 118, 119)	
Mass Gatherings (e.g., graduation ceremonies, concerts, sporting events) (120)	

**Often populated by workers who do not have access to regular medical care or testing sites or fear repercussions associated with authority figures (e.g., deportation).*

due to human error or malintent such as an act of terrorism. Human error involving personnel working in high containment laboratories and poor biosecurity practices could lead to an accidental release of a pathogen into the environment. Faulty facility management, followed by a series of other major engineering control failures, could lead to negative pressure laboratories becoming positive pressure and resulting in a pathogen release. Intentional acts of bioterrorism could cause disease outbreaks as well. While the US Military, CDC and USDA (85) publish lists of biological warfare agents (BWAs) and Select Agents, with the growing popularity and ease of access to commercial-off-the-shelf synthetic biology laboratory kits, it is possible that one could weaponize a relatively benign microbe without much investment of time or money. Even without modifying a microorganism, acts of bioterrorism could be committed simply through strategic release of influenza or another common pathogen which would result in the destabilizing of the community.

Depending on the training aid and methodology utilized, it is possible to train BMDDs to search for infected patients, the etiological agent itself, the facility growing (biomanufacturing or culturing) the pathogen in the case of terrorism, or even odors associated with the production of the pathogen such as spent culture or growth media. It should be noted, however, that the process by which the breadth and specificity of these capabilities is accomplished is quite complex. Training a BMDD has many similarities as the training process for an explosives or narcotics detection dog; however, there are some unique considerations that must be made before, during, and after canine selection, training, and deployment. The topic of canine selection and performance considerations has been reviewed in depth by Lazarowski et al., MacLean et al., and others (86–92). Training a BMDD differs in the following ways:

- Typically requires numerous potentially infectious patient samples and/or a potentially hazardous training aid that requires specialized containment.
- Presumed that the odor of disease or a pathogen is not the salient odor in the scent picture, therefore training must be more nuanced to teach the dog how to discern the signal from the noise and normal from abnormal.
- Canine threshold must reach lower limits of detection as disease/pathogens produce less odor than most common canine training aids (e.g., narcotics and explosive training aids).
- PPE is often required during training aid handling and storage.
- PPE is often required during deployment.

Finally, the deployment concept of operations, the medical and legal ramifications of a BMDD alert, and how to handle discordant results between BMDD and diagnostics should be determined before utilizing a BMDD operationally.

Deployment Scenarios

There are several ways in which BMDDs can be deployed during an outbreak. **Figures 2A–C** illustrates three of the primary ways in which BMDDs can screen humans for disease. The first scenario (**Figure 2A**) demonstrates a BMDD search of patient samples in a lineup. This set up has the least number of distractions for the BMDD as the search consists of discrete sampling points in the scent cans, a static odor presentation (i.e., the odor is not moving on a person in transit) which gives the BMDD adequate time to sample (sniff) the odor, and allows the sample collection team to reliably and reproducibly capture a sample from each patient/person. This scenario is the least hazardous of the deployment options as the BMDDs can be stationed in a separate room within the facility (e.g., airport, hospital, federal building) so there is no direct contact between the canine team and the public and/or the canine team and the patient samples. This scenario also eliminates potential allergic reactions to canines and interactions with people who fear canines.

The second scenario (**Figure 2B**) illustrates live human screening in a controlled manner in which people are individually searched by a BMDD behind a mesh screen/barrier. The humans individually enter a small room that is divided in half by a mesh screen barrier, the human is on one side and the BMDD is on the other side. Air flow would be established to flow from the human side to the canine side. The human is sampled or sniffed through the barrier and then leaves the room. This set up allows for physical separation between the patient and the BMDD while

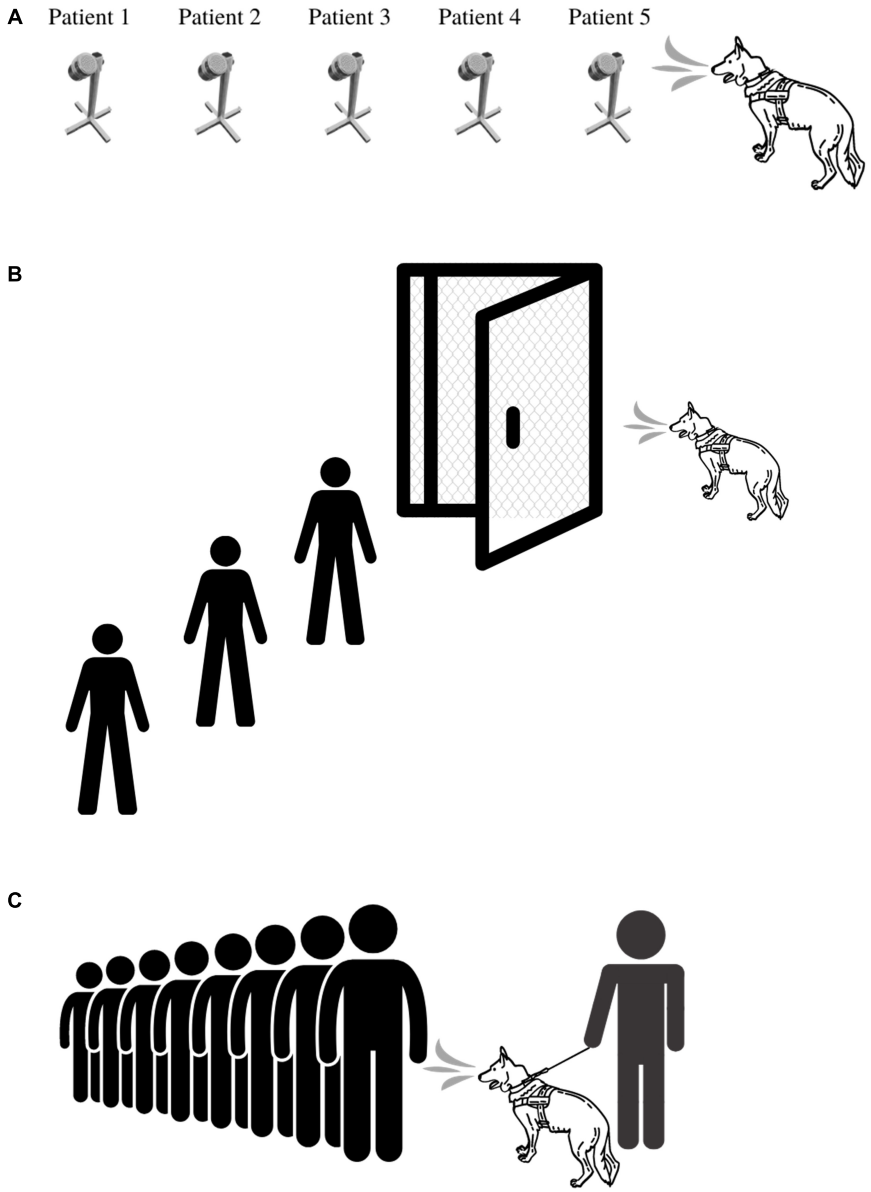

FIGURE 2 | BMDD deployment scenarios utilized during COVID-19 pandemic. **(A)** Deployment Scenario 1 illustrates the most basic of deployment scenarios in which a BMDD screens people or environmental samples in an area separated from the disease outbreak. **(B)** Deployment Scenario 2 illustrates BMDD people screening in the disease outbreak area, yet physically separated from the population. Here, the BMDD is separated by a mesh screen or high efficiency particulate air filter if needed and can screen people through a checkpoint or individually through a lineup or room. **(C)** Deployment Scenario 3 illustrates the most complex deployment scenario in which a canine team screens people either en masse or in a lineup by being able to directly sniff each individual or group of people.

providing the entire human as an odor source for the BMDD. The scenario also maintains more control of the operational environment by limiting distractions and controlling airflow.

The third scenario (**Figure 2C**) is the most difficult deployment scenario of them all as limited control of the operational environment exists and therefore BMDDs do not obtain the same sample from each person screened. Indeed, utilizing the first BMDD scenario, three COVID-19 detection studies had pooled sensitivities and specificities of 0.88 (95% CI, 0.84-0.91; I2, 85.3%) and 0.99 (95% CI, 0.99-0.99; I2, 97.4%), respectively (82); however, when one research group attempted to utilize the third deployment scenario, the positive predictive value plummeted to 28.2% (59). Jones et al. discuss the intricacies of screening travelers, specifically for COVID-19, in their perspective paper, "Could bio-detection dogs be used to limit the spread of COVID-19 by travelers?" (93) calling for additional research while also discussing plans for the next phase of their study in which sensitivity and specificity of BMDDs will be assessed at COVID-19 test centers where they can sniff individuals waiting to donate swab samples for formal diagnosis.

This study will provide much needed data to the body of literature for this difficult deployment scenario.

In general, one should first consider the deployment zone and whether the BMDDs will have direct person/patient contact, be segregated by a physical barrier that still allows for scent detection or be kept in a room used exclusively for scent detection lineups. Choosing the deployment method should further take into account a thorough risk analysis (to include legal and medical ramifications), the culture of the people being screened, how to sample people who are allergic or fear canines, and public perception and acceptance of canines (52). Two of these BMDD deployment methods were utilized during the COVID-19 outbreak. Private companies within the United States deployed canines at sporting events (**Figure 2C**) and the United Arab Emirates (UAE) utilized canines in airports keeping the canines in a dedicated sample screening room away from travelers (**Figure 2A**).

Once a patient sample or person is alerted on by the BMDD, if one is available, a diagnostic test should be performed to confirm the BMDD's detection response; however, it should be noted that the BMDD may have detected an earlier stage or asymptomatic presentation of infection that the diagnostic test will not be sensitive enough to detect.

Safety Considerations

Several safety considerations should be made before, during, and after the utilization of BMDDs. Specifically, a safety hazard analysis should be conducted to weigh hazard probability vs. hazard severity and create a decision matrix in which the overall risk of the operation (i.e., BMDD deployment) can be characterized. In this matrix, hazard probabilities range from unlikely, to seldom, occasional, likely, and frequent, while hazard severities range from negligible, to moderate, critical, and catastrophic (94). Pre-deployment medical screening of canine and handler, periodic testing (antibody and/or antigen) of canine team, and a system in place for daily monitoring of clinical signs, should all be established and maintained. While the human or canine may not be the ideal host initially, during an outbreak as pathogens mutate, pathogen host ranges may expand, hence the importance of ongoing disease screening of the canine team (dog + handler).

Part of risk management is developing and implementing controls. Controls should be evaluated during the decision-making process and implemented in several areas along the way toward a deployed detection capability. Depending on the type of disease outbreak, one may not have the option of deciding whether or not they are going the route of developing a BMDD as the nature of the etiological agent may dictate this path. For example, if faced with a highly pathogenic avian influenza outbreak that was capable of infecting birds, pigs, humans, and dogs, this pathogen would most likely be classified as a biosafety level four (BSL-4) organism, the handling of which would be limited to just a few dozen laboratories between North America and Europe. It is highly unlikely in this scenario that the medical community would have the resources to be supporting the canine community with patient or virus or virus-derived samples, and that the public health community would allow potentially infectious material to leave high containment laboratories. One factor working in favor of BMDD training aid creation, however, was published by Jendrny et al. when they observed that chemically inactivated (beta-propiolactone) SARS-CoV-2 saliva, urine, and sweat clinical samples could be used BMDD training and subsequently the dogs could generalize their detection capabilities to non-inactivated clinical samples and even to other body fluids (66).

Training Aids

Zoonotic outbreaks, however, should not *necessarily* preclude the development of BMDD training aids and eventual BMDD deployment. This is due to the fact that risk mitigation steps such as the aforementioned deployment scenarios, containment of the aid in the SciK9 (Lorton, VA) training aid delivery device (TADD), and/or odor ad/absorption-based training aid technologies, can be used in conjunction to mitigate risk and create a safe-to-handle training aid regardless of the hazard-class of the etiological agent. **Figure 3** illustrates the hierarchy of choices from most (top) to least (bottom) hazardous for the selection and development of canine training aids for BMDDs. Ideally, the outbreak pathogen would be categorized in the lower half of this diagram. It is important to remember that the premise of BMDD technology relies upon canine detection of odor, i.e., the VOCs emanating from the source and not physical contact with the actual pathogen or samples from a diseased human. Thus, by instituting proper safety and risk mitigation strategies, BMDD deployment may still be an option scientifically and operationally regardless of infectiousness of the disease outbreak pathogen.

In the disease outbreak scenario, BMDD training aids are typically divided into two categories depending on if the goal is pathogen detection as in the case of a biological detection dog (BDD), or disease detection as in the case of a medical detection dog (MDD) (**Figures 2A–C**). Training aids developed for the former can potentially consist of purified pathogen, pathogen culture, cell culture supernatant, spent cell culture media, inactivated pathogen (*via* heat, steam autoclaving, or chemical inactivation methods), modified pathogen (*via* utilization of existing vaccine strains, genetic engineering, or attenuation through passaging), and biomolecular components or metabolic products of the pathogen that produce a representative signature pathogen-specific odor (proteins, oligosaccharides, metabolites, envelope or membrane-associated lipids).

Training aids developed for MDDs can consist of direct capture of bodily fluids (e.g., urine, blood plasma, blood serum, sputum, nasal swabs, saliva, feces) or human scent (breath, sweat, skin/body odor) captured onto a substrate (clothing, gauze pad, cotton ball, worn surgical mask) from infected and uninfected patients (65, 77). The canine training aid(s) selected for a BMDD is of utmost importance as this decision will affect the canine's ability to detect the target odor (either disease or pathogen) and may influence the canine's ability to generalize to other target odors, e.g., novel patient samples, and discriminate from other similar pathogens, e.g., non-pathogenic strains of a virus or bacteria, both highly desirable BMDD skills. BMDD training aid selection, development, shelf-life, service-life,

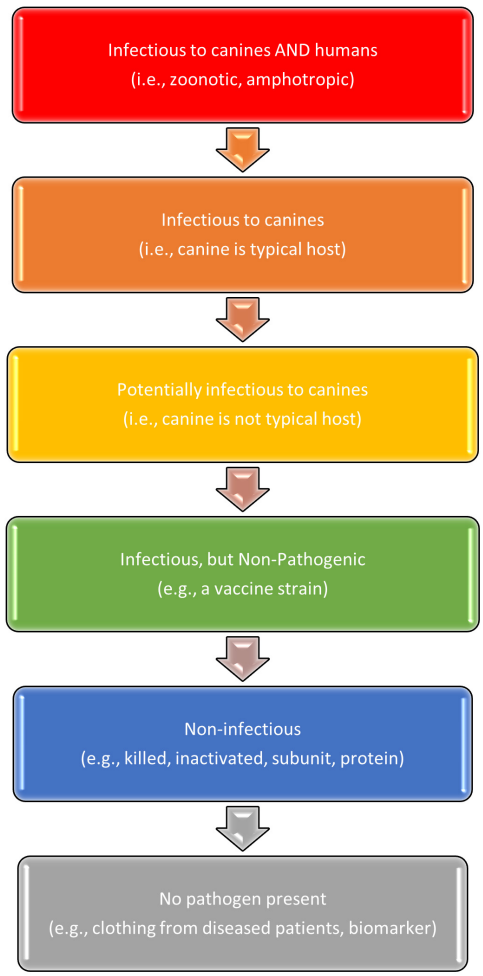

FIGURE 3 | Selection of canine training aids for biomedical detection dogs.

comparative analysis of efficacy and efficiency, and associated training methodologies and standards, and are all areas in dire need of research as each scientific group around the world took disparate paths in their approach to developing a COVID-19 detection dog capability.

Containment

The training aid delivery device (TADD) by SciK9 (**Figures 4A,B**) is a primary containment system for canine training aids that physically secures the training aid substance inside while allowing the odor out through a gas-permeable membrane (95). The TADD's membrane has hydrophobic and oleophobic qualities that allow for liquid and solid training aids typically required of BMDDs, such as blood, urine, or feces. Meanwhile, the TADD's membrane holder protects the membrane from physical penetration by the dog or handler as well as protecting the training aid from the operational environment of canine training, thus protecting precious clinical samples such as biopsy tissue or oropharyngeal swabs. The TADD facilitates the training of BMDDs on potentially hazardous materials such as their training aids in a safe manner while also protecting the handler, trainer, and the BMDD during the training process. The TADD was utilized during the training of COVID-19 BMDDs to protect the dogs from potential exposure to SARS-CoV-2 and any other pathogens that may be present in the patient samples (76). Another group tested the TADD for its ability to contain the SARS-CoV-2 virus by swabbing the outside of TADD-membranes after each day of canine testing and performing RT-PCR-assays to exclude virus escape (66). The TADDs demonstrated were swabbed and tested sixty-eight times, which resulted in 68/68 negative PCR reactions, thus demonstrating 100% containment of the hazardous biological material (66), while also enabling the BMDDs to successfully train on the odor of COVID-19 and detected non-inactivated saliva samples with a diagnostic sensitivity of 84% and specificity of 95%. Furthermore, in subsequent experiments the BMDDs were able to detect three non-inactivated body fluids with similar accuracy, achieving a diagnostic sensitivity and specificity of 95 and 98% for urine, 91 and 94% for sweat, 82, and 96% for saliva, respectively (66).

Odor-Ad/Absorption Based Training Aids

Odor ad/absorption-based training aids present a safe and reproducible way to capture and release a variety of odors from potentially hazardous material. From the traditional odor soaks utilizing natural fibers like cotton balls or towels to the latest polymer-based absorption training aids, these substrates allow training aids to be transported, stored, and handled without special requirements or permits typically mandated when dealing with hazardous material, and because they only contain the odor of the pathogen or disease state and are not infectious in nature, they present a safe alternative to training dogs to detect potentially deadly pathogens.

National Institute of Science and Technology (NIST) pioneered the polymer-based adsorption canine training aid work in their development of a polydimeythlsiloxane (**PDMS)-based training aid** for explosives (96, 97). The PDMS-base training aids (**Figure 5**) are non-toxic, non-infectious, and can be impregnated with nearly any odor, making the potential for this training aid technology nearly limitless for BMDDs. Based on the published research demonstrating the steady odor release rates of explosive material over time and recent method development publication on the rate of odor capture for less volatile targets (98), it stands to reason that this technology should be investigated for PDMS applicability in the creation of biological training aids.

Getxent tubes (**Figure 6**) represent another odor-absorption technology for the creation of canine training aids. The small tubes with an outer diameter of 8.0 mm, inner diameter of 5.4 mm, and length of 35 mm, are made of a proprietary blend of copolymers (certifiable biocompatible USP class VI), containing both polar and non-polar blocks allowing the absorption, storage, and release of odorous molecules (VOC) (99). They are supplied odorless and can be impregnated with target odor by co-incubating the Getxent tube within the headspace of the substance of interest. Getxent tubes were utilized extensively during the COVID-19 pandemic for quickly sampling the axillae of COVID patients during the research phase of canine training

FIGURE 4 | Training aid delivery device (TADD) breakout diagram **(A)** and photograph **(B)**.

FIGURE 5 | PDMS based Odor-Ab/adsorption canine training aid depicted in a small metal sniffer tin.

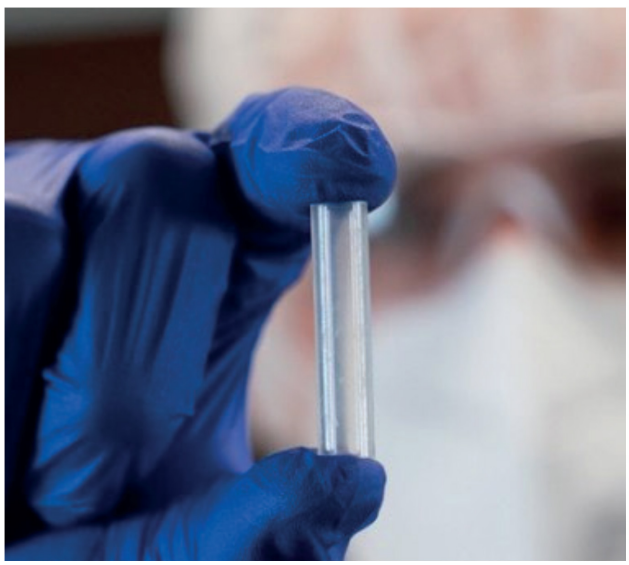

FIGURE 6 | Photograph of getxent odor adsorption tube.

and while screening the traveling public during the BMDD deployment phase (74).

Traditional odor-soaks may also be considered and have been demonstrated as efficacious in explosives, narcotics, and many other areas of canine detection. This method involves impregnating target odors *via* co-incubation in the headspace of the substance of interest with laboratory-grade glass, cellulose, or paper microfiber papers (**Figure 7**) (approximately 25–40 mm diameter pieces recommended) (100).

For all of the odor ad/absorption-based training aids, if the training aid is infectious or hazardous in nature, it is recommended that an appropriately sized filter barrier be placed between the training aid (e.g., pathogen (virus, bacteria), diseased tissue, field sample) and the odor-soak substrate to prevent accidental contamination of the substrate with aerosol or particulate from the potentially hazardous sample.

Safety Measures and Decontamination

Other protective measures that should be considered based upon a thorough risk assessment is the utilization of canine and human PPE. The same hierarchy of safety and health controls for healthcare personnel (**Figure 8**) should be applied to biomedical detection dog teams. BMDD handlers should receive proper education and training with respect to the pathogen or disease they are being asked to detect as a canine team. At a minimum, trainers and handlers should be able to recognize signs of disease, understand modes of transmission, any PPE required, and how to respond to a potential exposure. This education and training will help keep canine teams safe during training and operations. Industrial hygienists, professionals specializing in environmental and occupational health and safety, should be consulted to assess the engineering controls that can be instituted and should be emplaced. Industrial hygienists can also help develop workplace practice controls such as when and how to wash uniforms/clothing or the prohibited actions such as smoking, eating, and drinking in the work environment.

The biological risk assessment will help determine the proper PPE to utilize in addition to the appropriate work practices and containment requirements. This part of the risk assessment considers the properties of the biohazardous material such as pathogenicity, infectious dose, host range, agent stability and viability in the environment, availability of preventative therapies (e.g., vaccines), availability of post-exposure prophylaxis (e.g., immunoglobulin therapy), potential outcomes of exposure, and routes of exposure (inhalation, ingestion, dermal, or injection).

It should be noted that before BMDD utilization, both the canine and human should have individual risk assessments, followed by a joint canine team assessment. This approach will ensure that the canine-specific risks are being evaluated and avoid an incomplete or anthropocentric risk assessment. Canines face different risks than humans during operations. For example, canines are lower to the ground and will encounter different exposure hazards, as they are unable to utilize face-filtering respirators and masks whilst performing their scent detection duties, and rely entirely on the handler to keep them safe, having no concept of the risks involved.

Decontamination of the canine team and any associated equipment should be considered and planned for in advance of deployment. The effects of serial decontamination of canines are unknown at this time, so great care should be taken to avoid compromising the skin barrier by repeatedly washing or wiping canines. Only veterinary approved solutions should be applied to canines in an effort to avoid irritating or damaging canine skin and mucosa and avoid any potential toxicity issues. Dr. Erin Perry at Southern Illinois University published guidance on how to effectively decontaminate canines using two different methods, one in which water would be freely available and the other wherein water would not available and thus wipes are used to decontaminate the canine (101). The wipe-down procedure utilizing dilute povidone-iodine scrub wipes was later validated as the superior method for removing generic aerosolized particulate from canine coats when compared to dilute chlorhexidine-gluconate scrub wipes or water (102). While these studies provide information on the bulk removal of aerosolized particulate, it is still unknown if the chemicals in the decontaminant solutions are able to neutralize chemical or biological threats on canine fur as these decontamination solutions were developed and optimized on and for bare skin with the human end-user in mind.

Currently, Chemical Biological Radiological Nuclear (CBRN) decontamination recommendations for military working dogs are vague, lack a standardized protocol, and fielded kit, and are based upon the obsolete Army Field Manual 4–02.18 from 2004 (103). The U.S. Army's Public Health Center is currently conducting studies to address this knowledge gap. In conjunction with the US Army's Combat Capabilities Development Command—Chemical Biological Center (DEVCOM CBC), toxicologists, microbiologists, canine subject matter experts, and decontamination scientists are working together to validate the efficacy of two decontamination methods, the established method for chemical threats from Army Techniques Publication (ATP) No. 4–02.85 requiring access to water and a field-expedient low-water approach utilizing wipes and microfiber cloths (104). It is important to note that the military research and guidance will likely be directed toward acute exposure scenarios and decontamination and medical management of military working dog casualties. This is in contrast to the chronic exposure scenarios likely to be encountered by search and rescue (SAR) canines that Dr. Perry addresses in her decontamination guidance.

Due to the often disparate canine decontamination recommendations, it is therefore advisable to create a strategy for how personnel will handle potential exposure scenarios to both themselves and their canine partners and what measures should be taken on a daily basis to ensure that the canine does not become a fomite or disease transmission source.

The variance in how canine training is approached from trainer to trainer, amongst academic institutions, between countries, and from one detection discipline to another is staggering. One of the first research needs for BMDDs is standardization. If BMDD-based detection is going to have a role in the next disease outbreak scenario or a role in future medical diagnostics, there need to be established standards. Edwards et al., in their 2017 publication entitled "Animal olfactory detection of

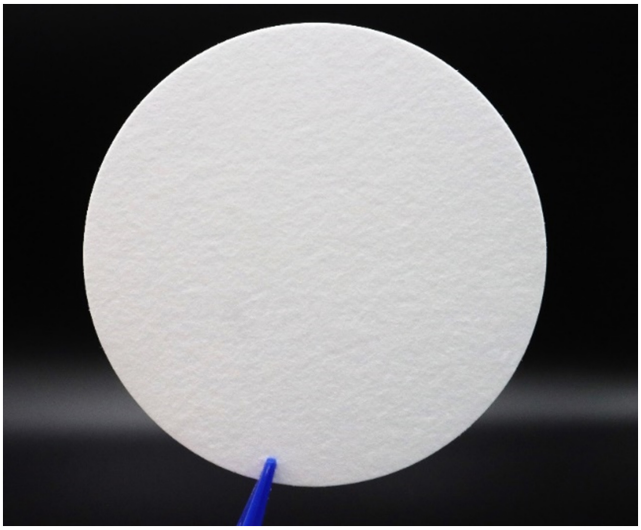

FIGURE 7 | Cellulose microfiber based filter paper for odor soaks.

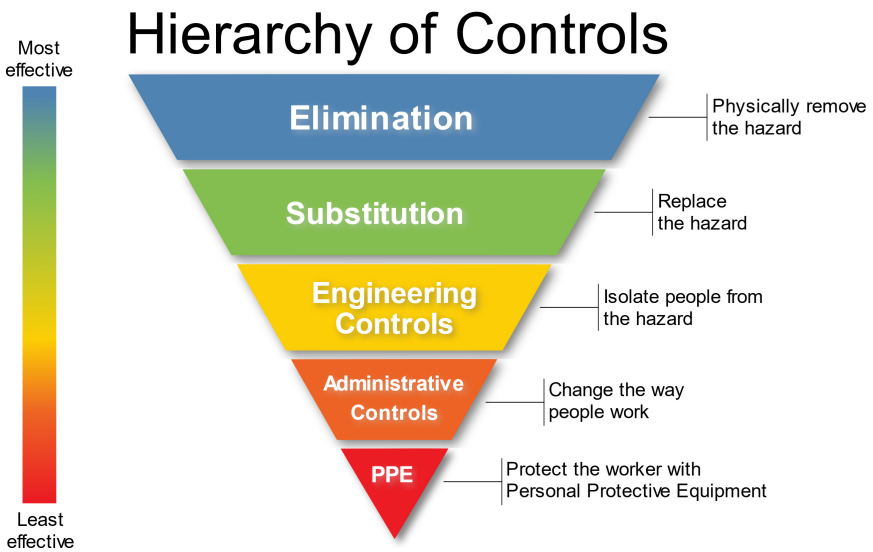

FIGURE 8 | Hierarchy of Controls in the Environmental Health and Safety Paradigm. Attribution: Original version: NIOSH Vector version: Michael Pittman, NIOSH's "Hierarchy of Controls infographic" as SVG, CC0 1.0.

human diseases: Guidelines and systematic review," outline the ideal training, testing and operational conditions for working with BMDDs and the associated samples (19). The authors provide recommendations regarding the type and depth of information that should be included when describing BMDD research so that these studies can help the scientific community compare the utility of detection methods for specific diseases or pathogens (19). Then, standards need to be brought to the human medical, diagnostic, public health, and regulatory communities to validate that the consensus publication standards will meet the evidentiary needs of a new detection/diagnostic-based technology.

From a training perspective, canine trainers should be prepared to deal with frequent and periodic quality checks and assurances on their BMDD. This is necessary because canine generalization in the operational environment works both for and against the detection capability. Generalization capability can potentially allow BMDDs to detect different disease clinical signs, different strains of a pathogen, asymptomatic or pre-symptomatic presentations, and diseases regardless of the patient age, sex, race. Yet generalization could also go beyond the detection capability we desire and potentially lead to BMDDs detecting related but non-pathogenic microbes, unrelated microbes, non-infectious disease presentations, non-specific

fevers, etc. Beyond certification, BMDDs will need to be recalibrated on their training aids often in order to ensure that they are still proficient on target odor. Additionally, BMDDs should also be certified using distracting odors from other diseases/pathogens to ensure that the dogs are still adequately discriminating target from non-target odor. Finally, during deployment, when the infectious status of a person is unknown, the reinforcement schedule of the BMDD must be carefully considered so as to not incorrectly reward the dog. There are several ways to address this, however, they are beyond the scope of this article.

Cooperation is also needed on the macro-level like the application of the One Health ideology wherein the veterinary and medical communities from the local to regional, national, and global levels communicate and collaborate to support for the research, development, test, and evaluation (RDT&E) needed to address current gaps in BMDD research, overcome BMDD deployment concerns, pair BMDDs with electronic sensors, and strategize how to scale-up operations for the next pandemic.

Research Needs

Immediate research needs to be conducted on the shelf- and service-life of biologically derived and patient-derived canine training aids, methods of sample containment, storage, and preservation, and best practices for characterizing BMDD training aid samples. Further research is also needed to determine what the dogs are actually detecting. A pared down, process-of-elimination approach combining systematic headspace analysis combined with canine olfactometry such as the one present at Dr. Nathan Hall's Texas Tech University Laboratory (105) could lead to biomarker discovery, a VOC-responsive colorimetric sensory array (106), or to the understanding that BMDDs are capable of far more than we have realized and a significant investment in the understanding of odor has enormous potential not only for once in a lifetime pandemics, but for breath-based diagnostics in the primary case setting, detection of invasive pests in big agriculture, assessment of the human volatilome for stress, fatigue, anxiety, and many other use cases.

Another research need is a holistic view and comparison of the medical detection dog and biodetection dog approaches to a disease outbreak. For example, during the COVID-19 pandemic, nearly all BMDD research groups around the world took the MDD approach, training dogs to detect the disease state of COVID-19 using patient clinical samples as canine training aids (52). However, a private business within the United States pursued the BDD approach, training dogs to detect the SARS-CoV-2 virus using viral proteins as canine training aids (107, 108). The BDD strategy rationale provided by the private business was based upon a discovery made with agriculture detection dogs studying one of the most severe pandemics in modern times, huanglongbing (HLB) disease of citrus, caused by the bacterium *Candidatus Liberibacter asiaticus* (*C*Las). Gottwald et al. observed that canines were detecting *C*Las bacteria directly rather than only host volatiles produced in response to infection and demonstrated this when the detection dogs identified *C*Las-infected tobacco, periwinkle, psyllid insect vectors, and bacterial co-cultures (109). The BDD approach utilized by Gottwald et al. and subsequent deployment of COVID-19 BDDs by private businesses within the US warrants additional investigation especially as this method has some advantages over the MDD approach.

The MDD approach to detection during an outbreak requires intensive recruitment of suitable subjects of both positive and negative disease status, and samples that represent the infected population in frequency, age, sex, ethnicity, and overall health status (access to healthcare, comorbidities, etc.) (19, 82). The MDD approach also requires contact with and handling of infectious patient samples, rendering the samples safe either by physical, chemical, and/or containment means, training dogs on up to hundreds of these disease positive and negative samples, special storage conditions for the samples or constant access to single-use samples, and great coordination amongst personnel that are typically not co-located (i.e., hospital staff and canine trainers) (81, 82, 110). This approach requires follow-up to ensure that patient infection status has not changed, e.g., a previously negative patient whose sample was collected for canine training became symptomatic and then tested positive 48 h later. This scenario does occur and needs to be controlled as patient-derived canine training aid samples are heavily relied upon to be negative or positive. The MDD approach also requires that canine training aids have patient history and demographic data to accompany each sample so that a representative cross-section of the population can be surveyed and presented to the dogs. These canine training aid samples should be assigned a unique number and firewalled from the patient data, i.e., deidentified, to preserve the privacy of research participants. Sample and patient information should include the data outlined in **Table 4**. The information provided in **Table 4** could easily be adapted for MDD studies involving agricultural detection dogs and plant diseases in which similar information about the age, growth conditions, health, and disease status of the plant would be important to know.

The BDD approach, such as in the case of a SARS-CoV-2 detection dog (assuming the training aid is viral protein and is efficacious resulting in a detection capability on COVID-19 positive patients) could have several advantages. (1) Unlike most detection or diagnostic laboratory-based equipment, canines have the unique ability to generalize and expand their "library" of target odors. If there is enough similarity between the odor (not the nucleic acid or amino acid sequence) of the current training aid and the odor of the circulating strains, that would be sufficient for the canines to alert. (2) The VOCs produced by the human immune response to an infection will eventually become part of the scent picture to the operational biodetection dogs, thus, in addition to the training aid, they will also have odors "in theater" that could allow for a persistent and enduring capability. (3) Canines have an incredible ability to find the novel odor in a familiar environment, called neophilia, and are known to find and detect anomalies due to this phenomenon. One theory is that since the viral proteins are novel to the canines, they are easily detectable compared to the environment and background odor. This novelty/anomaly could supersede and overcome the protein differences caused by viral mutation. The proteins would continue to be classified by the canines as "within the same odor

TABLE 4 | Minimum sample and patient information recommended for biomedical detection dog studies.

Sample	Examples of additional information
Unique identifier	Ties sample to patient data in a way that no patient or sample information can be gleaned from the identifier.
Type	Sputum, urine, blood, culture, insect casings, viral proteins
Suspension	Buffer, glycerol, media, formaldehyde, formalin, none
Sample capture Substrate/matrix	Swab, cotton pad, odor-absorption, none
Duration of sample collection	Length of time ventilating a surgical mask, duration of an odor-absorption tube in a patient's axilla (armpit), or co-incubation time of a filter paper to create an odor soak with the target substance
Infectious status	Live, inactivated/killed (state inactivation method), attenuated, non-hazardous
Sample containment	Serum separator tube, metal sniffer tin, urine collection cup, TADD, glass jar
Odor contributing sources	Gloves, masks, permanent marker
Time, date of sample collection	14:00, 2020-12-30
Time, date of sample receipt	
Time, date of sample Analysis	
Time, date of sample Storage	
Time, Date of Sample K9 Testing	
Collection Setting	Home, Diagnostic Lab, Research Lab, Hospital, Doctor's Office
Collector	Person who collected the sample, e.g., Patient or medical professional's name
Transport method and conditions	Shipped overnight cold storage or ground transport at ambient conditions?
Storage conditions	Location, temperature, humidity, any other unique conditions (e.g., vacuum storage, with desiccant, segregated positive from negatives, etc.)
Patient demographic data	**Patient history data**
Date of Birth	Current disease status
Age Range	Confirmed test result(s) for disease of interest
Ethnicity	Type of test(s) performed
Race	Date of testing
Sex	Date of results
City, State	Date of results notification
Type of Housing	Current symptoms
(e.g., detached home, apartment, communal living, etc.)	
Cohabitation with animals	Chronic health conditions
Cohabitation with human and their disease statuses	Positive for disease of interest in the past?
	Vaccination status for disease of interest
	List of current medication
	Pregnancy status

family" as their training aid, such that the canines can generalize and alert to the proteins produced by the virus variants. (4) Since the training aid is laboratory-made, cultivated in cell culture and purified virus protein, it can be re-formulated, modified, and multiplexed to include additional strains, variants, and proteins.

Following the SARS-CoV-2 biodetection dog example, there are also limitations that should be noted. Assuming that the training aid is composed of antigenic spike proteins, these protein sequences are constantly mutating as the RNA virus evolves. This could then require continuous reformulation of the training aid to ensure the composition/odor is representative of the circulating strain(s) of the virus. There are potential limitations should the disease outbreak be caused by a prion, whereby the infectious material is itself a protein and perhaps any attempts at modifying the protein to render it non-infectious alters or obliterates the odor profile, thus rendering any training aid ineffective. Finally, the biggest drawback of this approach currently is that it was the unconventional path, pursued by a private company in one country, and not third-party evaluated, while comparatively the MDD approach was pursued globally and successfully demonstrated by well-established research groups and published in peer-reviewed journals, therefore the biodetection dog approach, at least for its utility in a human disease outbreak scenario, is higher risk and unknown at this time. Due to the potential advantages, however, the biodetection dog approach should be considered and compared to the MDD

approach so that as many detection tools as possible exist for the next disease outbreak.

One area for improvement is the development of consistent and communicated training and testing protocols, making it possible for external parties to understand how successful dogs are at detecting an odor in both laboratory and real-world situations. Basic sensitivity and specificity reports do not completely inform readers about the conditions at time of testing (111). Different distractors may cause increased false alerts, testing scenarios may be less controlled than training leading to reduced true positives, and lack of blinding for trainers or testers may cause artificially high detection rates. We recommend stating if handlers and/or test administrators/observers are blind to target locations, communicating the type and number of non-target odors, and providing tables of test results in addition to overall sensitivity and specificity numbers. The criticality of not only publishing detailed protocol information, but also noting and tracking this information for each training and testing sample was highlighted by Guest et al. in their publication "Subtle Aspects of the Processing of Samples Can Greatly Affect Dogs' Learning" (112). To summarize, six dogs were trained to discriminate between hospital-sourced target urine and externally sourced control urine believed to be processed and stored the same way. During initial testing, dogs displayed good accuracy with a mean sensitivity of 93.5% (92.2–94.5) and specificity of 87.9% (78.2–91.9). However, upon further testing, when samples included hospital-sourced controls, the dogs performance greatly decreased in specificity 67.3% (43.2–83.3). Upon further investigation, it was found that the two sets of samples varied in one critical aspect—sample processing. The hospital-processed samples were tested by dipping a urinalysis stick into the sample, while the externally sourced samples were tested by pouring a small amount of urine over a urinalysis stick. Dogs had learnt to distinguish the target samples aided by the odor of this stick. This highlights the importance of considering every aspect of sample processing, but also pertains to sample collection, storage, handling, shelf-life, and presentation.

CONCLUSION

BMDDs offer a mobile, autonomous, non-invasive screening approach that provide real-time detection results in an efficient, reagent-free, and cost-effective manner. Furthermore, BMDDs can rapidly screening large numbers of people, samples, or areas, with a high degree of accuracy. But the one thing that BMDDs do that none of the other traditional screening or diagnostic tools can do is locate the target odor, find the infected person, source the unique signature volatilome, or alert to the most minute signal of a biological odor amongst the vast array of biological noise present in the operational environment. This "find" function combined with the ability of BMDDs to quickly clear the non-diseased patients/area, makes the potential for BMDDs unmatched in a disease outbreak scenario.

The limitations to BMDDs are broken down into those that are inherent in any scent detection dog discipline and those specific to BMDDs in a disease outbreak scenario. BMDDs themselves are living beings with the need for defined duty cycles to account for rest, sleep, eating, play, and all of the other needs of a canine. While rare, BMDDs have "off" days and thus it is advisable to have more than one BMDD in critical screening situations. And for now, we consider BMDDs a "closed system" in that they do not provide identifying information as to what they are detecting and instead simply provide a yes/no alert. Before a BMDD is ready for deployment there has already been considerable investment into the breeding, genetics, working dog criteria selection process, early neurological stimulation, early socialization training, and that is all in addition to standard rearing, veterinary care, and odor recognition training. Once a BMDD is trained and ready for deployment, in any scenario where they would need to be on-leash, such as **Figure 2C**'s deployment scenario 3, the BMDD requires a skilled handler to work together as a team during people or area searches. There is a plethora of other potential limitations, but most can be overcome with additional training and therefore are not considered inherent to BMDDs.

The limitations specific to BMDDs in a disease outbreak scenario are numerous in that many boxes must be checked before it can be done responsibly. Getting to the point of BMDD deployment takes enormous amounts of intergovernmental cooperation, effort, and coordination from access to patient samples to the navigating the legal aspects of people searching. Taking the MDD approach requires enormous effort dedicated to patient recruitment, testing, follow-up, sample remediation, characterization, storage, and containment, and all together, these endeavors require massive amounts of documentation, animal use protocols, institutional review board approvals, and coordination amongst medical, veterinary, and canine training personnel. Finally, without certification standard(s) specific to BMDDs in place, it will be difficult to install BMDDs in a way that instills public trust in the true capability of these incredible animals.

The potential of detection dogs during a disease outbreak is that they offer a promising strategy to addressing a gap in detection; however, to reach their full potential significant research investment in olfactory sciences will be required and the dividends will be substantial as the scientific outcomes will impact medical diagnostics, electronic breath-based sensors in public health, and stand-off detection technologies for hazardous materials.

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A Novel Method for Training the Interdiction of Restricted and Hazardous Biological Materials by Detection Dogs

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The interdiction of restricted and hazardous biological agents presents challenges for any detection method due to the inherent complexity of sample type and accessibility. Detection capabilities for this category of agents are limited and restricted in their mobility, adaptability and efficiency. The potential for identifying biological agents through a volatile organic compound (VOC) signature presents an opportunity to use detection dogs in a real-time mobile capacity for surveillance and screening strategies. However, the safe handling and access to the materials needed for training detection dogs on restricted or hazardous biological agents prevents its broader application in this field. This study evaluated the use of a polymer-based training aid in a viral detection model using bovine viral diarrhea virus mimicking biosafety level 3+ agent conditions. After the biological agent-based odor was absorbed into the polymer, the aid was rendered safe for handling through a rigorous sterilization process. The viral culture-based training aid was then used to train a cohort of detection dogs ($n = 6$) to discriminate agent-based target odor in culture from relevant distractor odors including non-target biological agent-based odors. Following culture-based training, dogs were tested for generalization to aids with infected animal sample-based odors across five sample types (fecal, blood, nasal, saliva, and urine). Within the context of the polymer-based training aid system, dogs were successfully trained to detect and discriminate a representative biological viral agent-based odor from distractor odors with a 97.22% (±2.78) sensitivity and 97.11% (±1.94) specificity. Generalization from the agent-based odor to sample-based odors ranged from 65.40% (±8.98) to 91.90 % (±6.15) sensitivity and 88.61% (±1.46) to 96.00% (±0.89) specificity across the sample types. The restrictive nature for mimicking the access and handling of a BSL 3+ agent presented challenges that required a strict study design uncommon to standard detection dog training and odor presentation. This study demonstrates the need to further evaluate the utility and challenges of training detection dogs to alert to biological samples using safe and manageable training aids.

Keywords: detection dog, bio-agent detection, viral detection, canine, bio-detection, bio-threat detection

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INTRODUCTION

Biological targets of interest represent a category of complex and relatively inaccessible threats for which instrumentation and traditional test methods are limited in rapid adaptation, mobility, and deployability (1). Biological threats can be manufactured and deliberately dispersed or occur through natural outbreaks and spread rapidly without being detected in real-time. The first line of defense in the detection of biological targets necessitates a rapid mobile technology to direct support resources, such as law enforcement, security teams, public health professionals and laboratories, toward suspect areas, materials and/or individuals. Programs, such as those outlined in the recent 2021 National Blueprint for Biodefense, do not readily have the capability to detect biological agents in real-time and state "More than 5 years after we released A National Blueprint for Biodefense [2015], the United States remains at catastrophic biological risk," indicating a critical security gap (2). This was echoed in the 2021 Global Health Security Index stating, "…all countries remain dangerously unprepared for meeting future epidemic and pandemic threats" and cite real-time surveillance as a capacity of potential international concern (3). The 2015 World Organization for Animal Health (OIE) Biological threat reduction strategy where surveillance, early detection and rapid response of bio threats was identified as one of the most sustainable and effective means of protection outlined in their strategy (4). The real-time detection of biological agents would provide governments with instant intelligence that could prevent, allow for early interdiction and intervention in, or confine a biological threat through precision resource allocation.

Detection dogs are a valuable threat detection asset used across disciplines from traditional law enforcement targets, such as explosives and narcotics, to novel applications in medical and biological detection (5–9). In a previous study for viral detection using trained detection dogs, our group demonstrated a detection capability for bovine viral diarrhea virus (BVDV) in live culture and successful discrimination of that virus from similar viruses, i.e., bovine herpes virus (BoHV-1) and bovine parainfluenza virus 3, in live culture (10). This model for virus detection represents a biosafety level 2 (BSL2) agent that affects multiple species and has closely related viruses of foreign animal disease significance. BVDV is in the *Pestivirus* genus alongside classical swine fever virus and border disease virus and belongs to the *Flaviviridae* family which encompasses viruses of zoonotic concern such as yellow fever virus, Zika virus, Dengue virus, and West Nile virus (11, 12). It is reported that at least three-quarters of human emerging infectious diseases originate in animals and four-fifths of potential biothreat agents are zoonotic, meaning they can be transmitted from animals to humans (4). The use of this BVDV model as a known canine detection capability for use in this study to mimic a restricted and hazardous agent in the development of a canine training aid provides a robust means for proof of concept under operationally relevant conditions.

Accessibility and technical proficiency required with sample handling for biological agents of high significance, especially BSL 3+, limit the feasibility of applying traditional training techniques toward restricted and hazardous biological targets of detection. The biosecurity levels represent the associated categorization of risk and increasingly restrictive standards for access and handling. A BSL-2 agent is considered by the Centers for Disease Control (CDC) to represent a human-associated disease agent that poses a moderate level of hazard to the handling personnel and/or to the environment, including animals and requires special practices of limited access and containment measures (13). Advancement to BSL-3 classified agents represent indigenous or exotic agents that can result in serious or potentially lethal disease and requires severely restricted access to designated and approved facilities, qualified personnel and multiple containment measures (14). Alternative training materials that represent select chemical components of larger target odor profile have been used in other disciplines with detection dogs to overcome the limitation of access to hazardous or restricted materials but establishing a biological agent-based odor profile with current instrumentation sensitivities remains a challenge (15). Additionally, identifying peak compounds does not necessarily represent the relevant odor profile for canine learning and biological agent recognition as these are complex odor signatures and a combination of signals is likely more representative of a unique odor profile rather than a single isolated compound. The field of volatile compound analysis has expanded and made significant gains toward higher sensitivity. Odors are predominantly comprised of volatile organic compounds (VOCs) which represent a category of low molecular weight compounds that are volatile under normal conditions (16, 17). However, complex targets remain difficult to identify with a unique odor "fingerprint" and they are dynamic samples that can change over time (18). Therefore, selection of an easily reproducible primary odor target for use as a pseudo-training aid, which does not use the original true material for its production, presents a challenge and may be limited in operational relevance (19).

Recent studies with a polymer-based odor capture and release (POCR) training aid demonstrated its capability of presenting qualitatively the same target-based odor profile for explosives such as triacetone triperoxide (TATP), for use in detection canine training (20–22). This aid represents a non-pseudo alternative that uses the true material in its manufacture directed toward ad/bsorption of the full target odor profile (19) while eliminating the associated risks and hazardous of handling and use. This technology uses a polymer-based material to safety capture the odor profile of a target of interest, which holds application toward biological targets with complex odor signatures. This technology provides an option for the safe presentation of the captured odor to dogs for use in training. The nature of the polymer material suggests it can physically withstand sterilization. This is a critical step needed in a potential training aid against biological threats as it mitigates the associated risk of exposure or contamination to biological targets while concurrently maintaining an ability to access and handle the odor outside of a laboratory setting for use in training with detection dogs.

This study aims to evaluate the use of the POCR training aid technology with hazardous biological agents under BSL 3+ conditions with the model BVDV virus. Within the POCR training aid system, dogs were trained to discriminate BVDV

culture-based odors from relevant distractor odors and other non-target viral agent-based odors using the POCR training aid and were tested for generalization to POCR aids with infected animal sample-based odor across five sample types (fecal, blood, nasal, saliva, and urine) as a potential restricted and hazardous agent capability. We hypothesized that using the odor ad/bsorption strategies for the POCR training aid technology and sterilization procedures to mimic a BSL 3+ biological threat would provide proof-of-concept for a safe odor presentation method in canine detection training with restricted and hazardous biological materials.

MATERIALS AND METHODS

All activities were approved and monitored by the AUCVM Institutional Animal Care and Use Committee (IACUC #2019-3514). The AUCVM is an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited facility and Biological Use Authorization was granted by the Auburn University Institutional Biosafety Committee.

Cattle

A prospective study was designed to control for confounding variables present in naturally occurring disease. Self-controls (animal samples collected prior to infection) and the full spectrum of infectious disease course from incubation period to recovery were available for sampling. Thirty, approximately 12-month-old, 800 lbs (365 kg) steer calves obtained from Animal Health Research (AHR), Auburn University, were utilized in this study and maintained in isolated pastures at the North Auburn BVDV unit. Each pasture has a dedicated corral and covered work area with chute. Diet and husbandry were identical between the two groups. Virus isolation and antibody screening assays were performed on the calves that were available to use in the study. All cattle were negative on virus isolation for BVDV and seronegative to BoHV-1, and all BVDV group cattle were confirmed seronegative for BVDV.

Group 1 cattle (BVDV-1; $n = 20$) were housed in a pasture that was separated from the pasture housing Group 2 cattle (BoHV-1, $n = 10$) by at least 9 m. Cattle were acclimated to the pastures for 3 days, followed by collection of samples as described below beginning on day −5. On day 0, each animal was infected with either 5 mL of BVDV inoculum containing 10^6 cell culture infective dose 50% ($CCID_{50}$) of BVDV-1b AU526 per ml (Group 1) or 5 ml of BoHV-1-1 Colorado (Cooper) strain containing 1 x 10^7 $CCID_{50}$ per ml (Group 2). Viruses were propagated under identical conditions in minimal essential medium (MEM) with Earle's salts, containing equine serum, L-glutamine, sodium bicarbonate, penicillin/ streptomycin/amphotericin (PSF) and purified water. All cattle were inoculated by intranasal instillation using 1-inch plastic intranasal catheter tips attached to a 5 mL single-use syringe.

Samples were collected from each steer on days −5, −4, −3, −2, −1, 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 14, 21, and 28. Sample types collected on each sampling day included: blood, nasal swabs, salivary swabs, urine, and feces. For sample collection, cattle were restrained in a squeeze chute. Blood samples were collected for canine training and surveillance of viral infection by virus isolation. Blood collection was performed by venipuncture from the jugular vein utilizing a vacuum tube system consisting of a Sarstedt Monovette® tube, Sarstedt needle adapter, and an 18-gauge, 1.5-inch needle. A total of 25 ml of blood was collected from each animal in serum separator tubes and in tubes containing EDTA for eventual isolation of white blood cells. Nasal and salivary samples were collected by swabbing each nostril and mouth with separate sterile cotton-flocked swabs of approximate 0.69" tip length size. Samples were placed directly into sterile cryovials. Fecal samples (∼20 grams) were manually collected by inserting a gloved hand or fingers into the rectum, and then samples were placed into empty plastic cryovial containers. Additionally, urine was collected into sterile urine collection cups, when possible, either when the animal urinated voluntarily while in the chute or following gentle stimulation ("feathering") of the prepuce, then transferred immediately by pipette into a sterile cryovial. All samples from an individual steer were placed in separate sealed waterproof bags and placed in an ice cooler prior to transfer to −80°C storage within 4 h. Sampling and transport of materials was performed separately for BVDV and BoHV-1 groups. Strict biosecurity protocols were followed with full change out of personal protective equipment and order of entry for sampling, with collections occurring in Group 2 (BoHV-1) prior to Group 1 (BVDV) BoHV-1.

Throughout the study, animals were visually inspected daily for clinical signs of illness. Viral inoculations were expected to cause subclinical to mild clinical signs, including mild fever, upper respiratory signs (clear nasal discharge), and reduced appetite. Animals were inspected daily by animal health research personnel and were examined by veterinary staff on dates of collection −5, −4, −3, −2, −1, 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 14, 21, and 28 in conjunction with collection of blood samples for virus isolation (VI). On days 0 and 28 blood was collected for virus neutralization (VN). Steers were allowed full recovery and maintained within the AHR herd following the study.

Virological Assays and Cultures

Culture

Growth of BVDV for culture-based training aid development contained 1 x 10^6 and 1 x 10^5 cell culture of BVDV-1b AU526 (BVDV group) and BoHV-1 Colorado (Cooper) strain containing 1 x 10^7 in minimal essential medium (MEM). Culture preparations were made using previously described methods (10).

Virus Isolation

Detection of BVDV was performed in buffy coat cells from whole blood samples of all cattle (BVDV and BoHV-1 groups) through co-cultivation with Madin-Darby bovine kidney (MDBK) cells in adaptation of previously described methods (23). Briefly, the buffy coat was reconstituted to 1mL total volume in MEM with 10% EQS (media) and layered over cells that had been seeded 24 h earlier in a 24-well plate. Following three freeze-thaw cycles to release intracellular material, lysates from this procedure were incubated for 72 h on MDBK cells and assayed in triplicate by

an immunoperoxidase monolayer assay using the BVDV-specific monoclonal antibodies D89 and 20.10.6.

Virus Neutralization

Sera were separated from clotted blood following collection, heat inactivated at 57°C for 30 min, and stored at −80°C until analysis. A standard virus neutralization microtiter assay was used for the detection and quantification of BVDV antibodies in sera of all cattle, as previously described (24). Sera were tested for neutralizing antibodies using the BVDV cytopathic strain NADL. Testing of sera for antibodies against BoHV-1 (BoHV-1 group) was performed using the BoHV-1 Colorado strain as previously described (25).

Training Aid Development

The POCR training aids were prepared for use in biological detection using a method similar to those previously described for explosives odor capture (22, 26) with biological target-specific modifications for sterilization, patent pending (27). Odor profiles were "charged" onto the polymer material in a biosafety hood using standard laboratory clean technique. This included wearing disposable nitrile gloves and aliquoting materials with sterile disposable pipette tips onto clean glass petri dishes for charging. This charging involved placing the training aids in proximity to, but not in direct contact with, raw materials to ad/bsorb VOCs emitted by respective targets or distractors. The aids were removed after the charging process and placed through a rigorous two-step, high heat, high pressure sterilization process consistent with biosafety protocols for restricted agents (13). This procedure was utilized to conduct the experiment under the most stringent circumstances for rendering a POCR that has been exposed to a biological agent safe for training. The sterilization process was performed under these conditions to serve as model for use of protocols and materials relevant to restricted and emerging agents. Training aids for initial training and baseline performance were made with cultures and training aids for testing and probing were made with nasal, salivary, blood, fecal and urine samples collected from days +6 to +10 as it represented the peak infective window.

Contamination Risk Assessment

A set of culture POCR training aids were made for contamination risk assessment. The POCR training aids were "charged" fresh in identical fashion to the training aids used in canine trials. To represent the highest level of risk, aids were sampled for possible surface contamination pre-sterilization. Virus was propagated from original stock culture. Two swabs were moistened for each plate, one with 1 ml PBS in a collection tube and the second with 1 ml media in a collection tube. Each swab was used to sample the entire surface of the POCR and placed into its respective tube resulting in 20 samples for BVDV (10 for each phosphate-buffered saline (PBS) and media, respectively) and 20 samples for BoHV-1 (10 for each PBS and media, respectively). Subsequently, 500 microliters of each tube were used for RNA/DNA extraction to perform qPCR detection, adapted from previously described methods (23). The assay utilized in this study involved use of a probe rather than SYBR green and using the QuantaBIO qScript XLT master mix. An additional 500 microliters were placed in −80°C for reserve pending positive VI testing follow up.

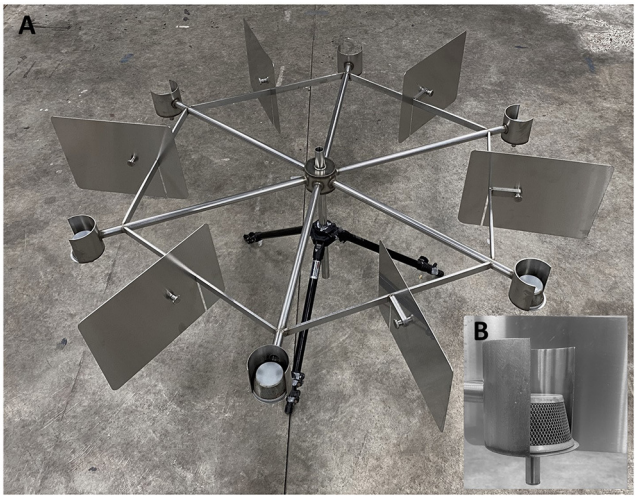

FIGURE 1 | Testing Wheel Image: **(A)** Stainless-steel testing wheel with 6 radial arms and sample divider plates between positions; **(B)** Walled (3/4 circumference) cups and wire basket for sample presentation.

Canines

Subjects

Six Labrador retrievers (2 M/4 F) between 1 to 5 years old (mean age: 2.35) from the Auburn University College of Veterinary Medicine (AUCVM) Canine Performance Sciences (CPS) detection dog program participated in this study. Dogs were housed in individual indoor/outdoor runs within the kennel complex at the AUCVM. The dogs were raised through the same breeding program and had similar puppy development and varying odor detection training experience prior to placement on the study. All dogs selected had no prior experience with biological or medical detection.

Training and Generalization Testing

Training and testing occurred in a 4 x 4 m dedicated biosafety (BSL 2) training room that was climate- and humidity-controlled. In the center of the room was a stainless-steel scent wheel (1.31 m in diameter) with six arms (**Figure 1A**). A stainless-steel cup attached to the end of each arm held samples for presentation (9.53 cm in diameter) (**Figure 1B**). Upon placement of a sample (i.e., POCR), the cup was covered with a wire mesh basket to allow odor sampling while preventing physical contact between the dog and the substance (**Figure 1B**). Dogs were familiarized on the task of performing a wheel search prior to start of study.

Test sessions were conducted single blind, with the dogs trained to work off leash. The handler always remained outside of the room out of the dog's sight (**Figure 2**). At the start of each trial, the experimenter placed the samples on the wheel and then exited the area, remaining inside a control room adjacent to the wheel room for the duration of the trial and viewed the dog through a one-way mirror. The handler then sent the dog

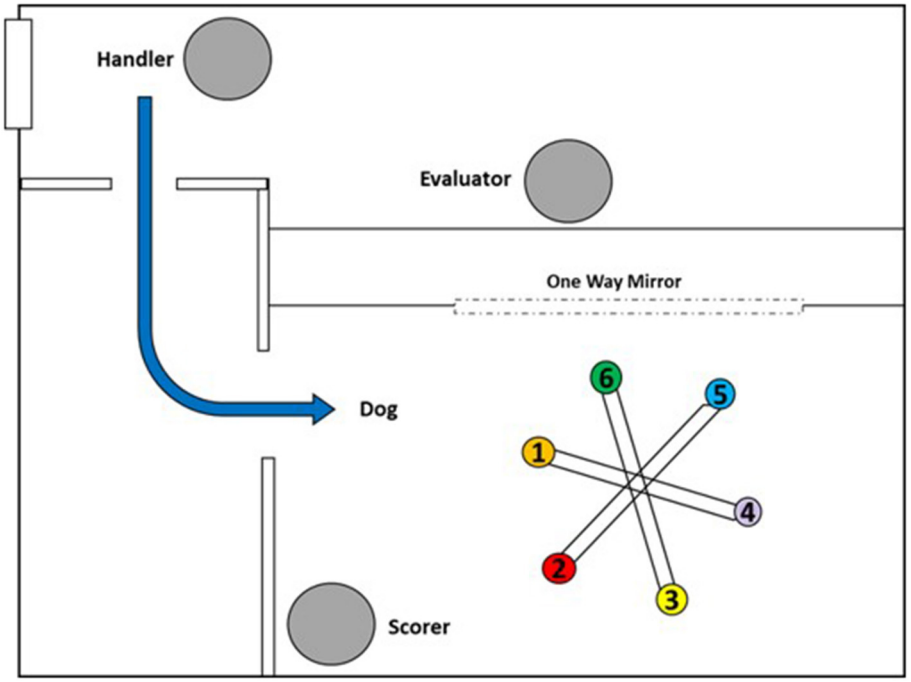

FIGURE 2 | Room layout for wheel testing. Scorer is unaware of trial set-up and blind to target placement or trial type and unable to see the moderator behind the one-way mirror. Dog is released from the handler to enter the wheel room off-leash and search independently.

into the room to sample the wheel. The experimenter signaled the outcome of each trial to the handler using hand signals only visible to the handler. If the dog made a correct indication (a sit response, operationally defined as full contact of the hindquarters on the ground for any duration in front of the target position (28) or searched the last position with no false alerts on trials with no target present, the experimenter signaled with a thumbs up and the handler recalled the dog and delivered a reward (play with a ball). If the dog searched all positions without making an indication when a target odor was present (false negative), the experimenter signaled with a thumbs down, and the handler called the dog out of the room without delivering a reward. The same call, "come" was used for calling the dog out of the room on all trials. If the dog responded at a position that did not contain a target (false alarm), the dog was ignored and allowed to continue searching the remaining positions. An observer who was blind to the presence and location of targets, positioned in the corner of the wheel room (**Figure 2**), scored whether and at which location dogs made a response.

Dogs were first trained to detect the odor of BVDV viral culture using the POCR training aid (see **Table 1**). Dogs were initially introduced to the odor using a standard odor discrimination line-up with stainless steel boxes within the same testing room along the straight corridor adjacent to the wheel, in which dogs were taught to associate the odor of the BVDV viral culture POCR with a reward (play with a ball) and to discriminate it from "blank" (i.e., uncharged) POCR. Training then progressed to the wheel scenario, culminating in a baseline session to serve as confirmation of the dogs' proficiency in detecting the trained target and to serve as a comparison with their proficiency in detecting the targets in the subsequent generalization tests. Number of training trials prior to baseline testing varied by individual dog based on chief instructor assessment of performance improvement and progression of odor learning. The baseline sessions were conducted over two consecutive days with 10 trials per session. Each session consisted of 6 target and 4 blank trials which were randomized across the session. The placement of targets was counterbalanced across the six positions, with distractors in all other

TABLE 1 | Culture POCR training and testing list. The list of target and distractors used in the training and testing of culture POCR.

Category	Sample
Target	BVDV Culture
Distractors	BoHV-1 Culture
	Media component: equine serum
	Media component: sodium bicarbonate
	Media component: antibiotic combination consisting of penicillin/streptomycin/amphotericin (PSF)
	Media component: L-glutamine
	Media component: minimal essential media (MEM) with Earle's salts
	Media component: purified water
	Media Whole

TABLE 2 | Sample POCR generalization testing list. Each set of targets and distractors were used across each sample type.

Category	Sample
Target	BVDV Sample Animal (days +6 to +10)
Distractors	BoHV-1 Sample Animal (days +6 to +10)
	BVDV Self-Control (days−5 to−1)
	BoHV-1 Control (days−5 to−1)

Six individual targets were selected each session at a 1:1 ratio with BoHV-1 sample and BVDV self-control as distractors.

positions. The non-target ("blank") trials contained all distractors. Distractors included blank media whole, each media component, and a non-target viral culture (BoHV-1) (see **Table 1**).

Generalization tests (see **Table 2**) occurred the same as the baseline sessions with dogs completing only one session per day. The first probe odor (nasal sample POCR) was presented across three consecutive sessions consisting of 10 trials each to determine dogs' ability to generalize from viral culture POCR to sample POCR. Each trial presented 6 target and 4 blank runs randomized across the session. Next, dogs completed eight additional sessions, two for each sample type on POCR, in the following order: saliva, blood, urine, fecal, urine, saliva, fecal, blood. Responses to probe odors were reinforced like baseline trials to minimize disruption of performance. If deemed necessary by the chief instructor based on individual dog's task focus, search behavior and number of elapsed trials with no reward, a baseline trial with culture POCR was inserted to maintain motivation. The distractors selected included self-matched controls (pre-inoculation) for positive target samples in respective sample types, clinically similar viral positive samples (BoHV-1) and controls (pre-inoculation).

Controls

All targets and distractors and their holding containers were changed after each trial. Baskets, basket holders, scent wheel apparatus, and POCR devices/petri dishes were only handled using nitrile gloves and metal forceps to eliminate human scent (29). Baskets and petri dishes were sanitized with high heat after each use in a commercial dishwasher (up to 68°C). All targets and distractors were handled by the same person to eliminate the dogs' ability to identify a person-scent associated with the categories of samples. All personnel present donned gowns, gloves and goggles while conducting experiments. Distractor odors were present in all non-target positions to serve as negative controls for calculating specificity/false alarm rate. Each trial included self-matched controls (pre-inoculation) for each individual steer that would be presented during the trial (6 targets from days +6 to +10 and 6 self-matched controls from days−5 to−1). Days +6 to +10 were selected for animal sample aid presentations as it represented the peak infective window. Target samples were only presented once for each dog in a given sample type, no target samples were repeated across the trials for any given sample type for any individual dog.

Performance Scoring and Data Analysis

On each trial, dogs' responses were scored as a true positive (response to a position containing a target), false negative (no response to a position containing a target), false alarm (response to a position not containing a target), or true negative (no response to a position containing a distractor). Sensitivity for each target was calculated as total true positives out of total exposures to the target, averaged across all dogs across all sessions for that target. Specificity was calculated as total true negatives out of total positions searched, averaged across all dogs across all sessions for that target. Generalized linear mixed effects models (GLMMs) were used to analyze sensitivity and specificity as a function of the fixed factor of sample type (culture, nasal, saliva, blood, urine, and fecal). Analyses were performed in the R statistical program (Version 1.2.5033, RStudio). Data represent the mean (± SEM) unless otherwise noted. Additionally, we separately report total responses across dogs to the first presentation of each sample tested. Origin (Pro), Version 2021b. OriginLab Corporation, Northampton, MA, USA was used for receiver operator characteristics (ROC) curve analysis. A subset of videos (two randomly selected sessions for each sample type) was scored by an additional blind observer and total recorded dog sits and position checks were used to calculate inter-rater reliability, which was very good for total true positives (ICC = 0.98, $p < 0.001$), true negatives (ICC = 0.99, $p < 0.001$), false positives (ICC = 0.99, $p < 0.001$) and false negatives (ICC = 100, $p < 0.001$).

RESULTS

Clinical Evaluations and Virological Assays

Following inoculation, cattle in the BoHV-1 group demonstrated clinical signs of infection at varying degrees across the course of infection to include hyperthermia and copious mucous to mucopurulent nasal discharge. Cattle in the BVDV group did not develop clinical signs of infection.

Virus neutralization results from all 20 BVDV-infected group 1 cattle demonstrated that all animals were successfully infected with BVDV, as indicated by a >4-fold increase from baseline on day 0 (1 ± 0 titer) to day +28 (62±19.6 titer) for BVDV. No measured increase for corresponding BoHV-1 results in group 1 cattle. The 10 BoHV-1 group 2 cattle demonstrated a >4-fold increase from baseline on day 0 (1 ± 0 titer) to day 28 (57.6 ± 9.29 titer) for BoHV-1 and no measured increase for corresponding BVDV results.

BVDV virus isolation results across all 20 BVDV group 1 cattle demonstrated viral detection in 16/20 individuals across a range from day +3 to day +10 with 2/20 individuals infected on day +3, 7/20 day +6, 13/20 day +7, 7/20 day +8, 2/20 day +9, 1/20 day +10 and 0/20 day +14.

Canine Training and Generalization Testing

Dogs completed 143 training trials on average, across ∼3 months, on the fixed sampling wheel. Baseline session confirmed that dogs were proficient in detecting the trained target (viral culture POCR), with 97.22% (± 2.78) sensitivity and 97.11% (± 1.94) specificity.

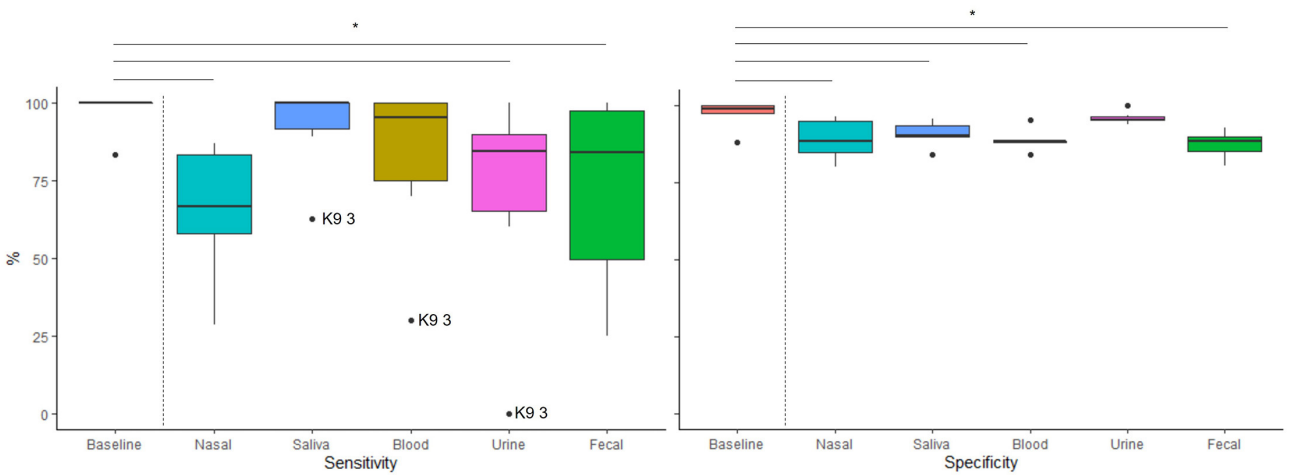

FIGURE 3 | Distribution of canine ($n = 6$) sensitivity and specificity for each target (Culture, Nasal, Saliva, Blood, Urine and Feces). Horizontal lines inside boxes represent medians, boxes represent the interquartile range (IQR), and whiskers represent the range of values within 1.5 X IQR. Dots represent individual dogs that were determined outliers (either less than the first quartile - 1.5 X IQR or greater than the third quartile + 1.5 X IQR. Horizontal bars above the graph show significant differences in performance between baseline and sample tested.

Table 3 reports sensitivity and specificity for the baseline session with culture and each of the tested sample types. Sensitivity and specificity for each tested sample type as compared to baseline is shown in **Figure 3**. Sensitivities for nasal, urine, and fecal sample types were significantly lower than baseline sensitivity (GLMMs: t (25) $= -2.88$, $p = 0.008$; t (25) $= -2.48$, $p = 0.020$; t (25) $= -2.27$, $p = 0.032$, respectively), with no differences between saliva or blood compared to baseline (GLMMs: t (25) $= -0.48$, $p = 0.634$; t (25) $= -1.41$, $p = 0.172$, respectively). In addition, sensitivity for nasal samples was significantly lower than for saliva (GLMM: t (25) $= -2.40$, $p = 0.024$). There were no other significant differences in sensitivity between targets ($p > .057$). One dog (K9 3) represented the outlier across three sample types (see **Figure 3**).

Specificities for nasal, saliva, blood, and fecal were significantly lower than baseline (GLMMs: t (25) $= -3.61$, $p = 0.001$; t (25) $= -2.84$, $p = 0.009$; t (25) $= -3.73$, $p = 0.001$; t (25) $= -4.28$, $p < 0.001$, respectively). However, there was no significant difference between urine and baseline (GLMM: $t(25) = -0.47$, $p = 0.631$). In addition, specificities for nasal, saliva, blood, and fecal were significantly lower than urine (GLMMs: t (25) $= -3.12$, $p = 0.005$; t (25) $= -2.35$, $p = 0.027$; t (25) $= -3.24$, $p = 0.003$; t (25) $= -3.79$, $p < 0.001$, respectively). Specificity across testing was above 90 % ($M = 91.43$, $SEM = 1.68$) (**Table 3**), indicating that dogs were discriminating the target virus from distractors.

Examining first-trial responses indicates that generalization varied by dog and across sample types. The probe presentation order in POCR was nasal presented across three consecutive sessions consisting of 10 trials each followed by eight sessions, two for each sample type, in the following order: saliva, blood, urine, fecal, urine, saliva, fecal, blood. For three sample types, first-trials responses were lower than the second trial responses (first trials nasal: 1/6, urine: 2/6, and fecal: 3/6; second trial nasal: 5/6, urine: 5/6, fecal: 4/6). In the other two sample types, all dogs generalized with high proficiency on first and second trials (first trial saliva: 5/6 and blood: 5/6; second trial saliva: 4/6, blood: 4/6).

TABLE 3 | POCR testing results. Average (± SEM) sensitivity and specificity by dogs for each odor tested.

Target	Sensitivity%	Specificity%
Culture	97.22 (2.78)	97.11 (1.94)
Nasal	65.40 (8.98)	88.88 (2.72)
Saliva	91.90 (6.15)	90.64 (1.67)
Blood	81.67 (11.38)	88.61 (1.46)
Urine	69.81 (15.01)	96.00 (.89)
Fecal	72.13 (13.11)	87.36 (1.77)
Sample average	76.18 (10.93)	91.43 (1.68)

Sensitivity and specificity of detection by dogs and by VI testing is graphically represented separately by the ROC curves in **Figure 4**. The ROC curve is graphical representation of the diagnostic ability of a binary classifier system plotting the true positive rate (sensitivity) in a function of the false positive rate (100-Specificity) and is a tool used for medical diagnostic test evaluation (30). Overall performance in blood POCR for each dog demonstrated K9 3 to have a lower area under the curve on the ROC analysis than VI in the cattle samples tested, while the remaining five dogs had a higher area under the curve than VI (**Figure 4**). All test curves demonstrated performance better than chance with respect to reference (dotted line **Figure 4**).

Contamination Risk Assessment

Contamination testing was conducted on 20 representative POCR training aids, 10 each BVDV and BoHV-1. Each POCR swabbed twice, once with PBS and once with media. Swabs were

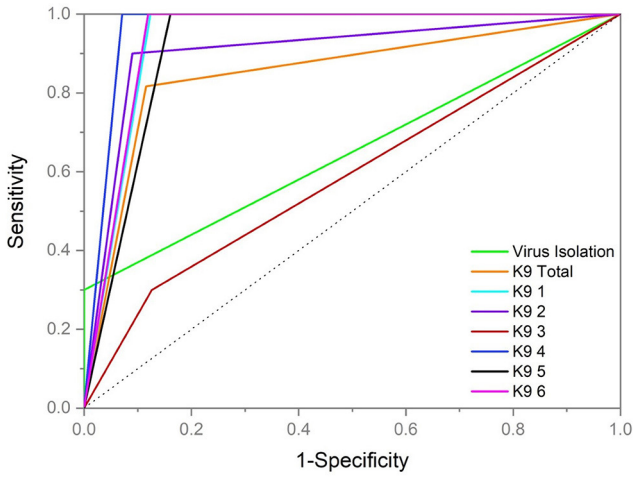

FIGURE 4 | Graph of Receiver Operator Characteristic (ROC) curve representing all dogs ($n = 6$) as a test modality (K9 Total), each individual dog (K9 1, K9 2, K9 3, K9 4, K9 5, and K9 6) and virus isolation monitoring. K9 results represent blood sample testing during peak infective window samples of positive day +6 through +10 and corresponding trial control samples examined by the dogs. Virus isolation results represent all blood samples for virus isolation testing during peak infective window samples of positive day +6 through +10 and control samples. The dotted reference line runs through the center representing a diagnostic performance no better than chance.

analyzed using qPCR and results indicated four aids, 2 BVDV with media (C_q=36.395 ± 0.007 SD) and 2 BoHV-1 with PBS (C_q = 36.905 ± 0.049 SD), contained trace levels of genetic material. The four swabs representing the four training aids positive for trace levels of genetic material on qPCR were subsequently analyzed by virus isolation. All samples were negative on virus isolation for presence of live virus.

DISCUSSION

This study used a model virus (BVDV) to demonstrate the utility of a polymer-based training aid to capture a biological agent-based odor profile for use in training and testing for sample-based odors. The results indicate that BSL3 decontamination and odor absorption strategies for the POCR training aid technology hold application toward biological detection and support the perceptive presence of a unique biological agent-based odor profile for BVDV distinguishable from another representative clinically similar virus (BoHV-1) across multiple sample types.

The unique biological agent-based odor profile perceived by trained detection canines in culture-based aids were shown to be recognizable, with varying rates of generalization, across the five sample types. All dogs met a high level of performance of over 97% sensitivity and specificity in the culture prior to testing with the samples. Detection rates to the tested samples ranged from a low of 65.40% sensitivity in nasal samples and 87.36% specificity in feces to a high of 91.90% sensitivity in saliva and 96% specificity in urine. These results indicate a moderate to high rate of confidence in the presence of a unique odor associated with BVDV culture that relatively preserved in aid development post-autoclave procedures. This unique odor is also shared across sample types (nasal, saliva, blood, urine, and feces). Further, that unique odor is also a commonality shared across separate BVDV positive individuals as each steer's positive sample type was presented during testing only once for each dog and stringent controls were used to prevent use of individual animal cues by the dog for target recognition.

Across all dogs, one outlier (K9 3) represented the highest contribution to variance. Individual variability in generalization by dogs has been reported (21, 31), which could be the result of numerous uncontrolled or unknown factors such as training history, age, or temperament. With a small sample size, this variability supports the need for further research to explore factors contributing to individual differences in generalization. However, these differences may underscore the exigent requirements of detection dogs to perform the complex detection tasks of biological targets across multiple contexts, which may result in a narrower criterion for dog selection in this field.

Generalization testing performed with culture-trained dogs across the five sample types was lowest to nasal samples (65%), which could be due to several unknown factors such as collected sample odor retention rates and sample type features (e.g., mucus), or a procedural factor such as testing order given that nasal samples were the first to be tested following culture training. The improvement in the second trial for nasal samples likely represents a challenge in transition of context for generalization from a culture-based context to a sample-based context. The change in target context, from culture to cattle sample, represents a variation to the presented odor profile and introduces additional background odors. With individual sample-based training, detection dogs have shown an ability to discriminate positive individuals from negative individuals based on individual scent vs. condition-associated odor, such as disease or infective state (32). To control for this, dogs were tested only once with any positive individual in a given sample type with no repeat exposures, Additionally, self-matched negative controls of the positive targets (i.e., pre-inoculation) were used as distractors within the same session. Thus, the use of self-controls and no repeat exposures suggests that this improvement is not attributable to individual-based sample learning.

The subsequent sample type tested, saliva, demonstrated that the relatively non-invasive sampling of saliva yielded high generalization on first trial responses across the six dogs at 5/6, which may indicate that, after an initial context generalization occurs from the culture-based to sample-based context, the subsequent rate of generalization for additional sample types improves. Overall, between the two non-consecutive sessions, dogs showed no significant differences in sensitivity on saliva from culture-baseline even with the outlier of K9 3. Additionally, the next tested sample (blood) yielded high generalization on first trial responses across the six dogs at 5/6. Overall, between the two non-consecutive sessions, dogs maintained high sensitivity not significantly different from baseline, but with a wider overall range across individual dogs with no single significant outlier compared to saliva. However, upon presentation of urine and

feces the rate of generalization during the first session dropped, though remained higher than the initial seen with nasal. The complex odor matrices represented in urine and feces increase the amount of background odor noise anticipated due to representing the two primary elimination pathways for bodily waste products. This likely presents a larger initial challenge to generalization that may improve on subsequent exposures due to context generalization.

Virus neutralization (VN) demonstrated a successful inoculation across all cattle with BVDV in group 1 and the virus isolation (VI) monitoring across multiple days demonstrated peak period of viral shed at days +6, +7 and +8. Using virus isolation as a screening tool was highly specific (100% ±0) with lower sensitivity during the expected peak window day +6 to +10 (30% ± 1). Though not a goal of this study to compare diagnostic capabilities the results of the virus isolation tests, which currently represent the "gold standard" in screening techniques for cattle, indicate that the dogs were more sensitive to positive cattle samples than VI. In the ROC curve analysis performed to evaluate these differences using the same sample type (blood), the dogs' results indicated an overall higher sensitivity (81.67% ± 11.38) but lower specificity (88.61% ± 1.46). Individual dog performance varied with one dog, K93, demonstrating an overall lower area under the curve value than virus isolation. The cattle results were VN-positive in all 20 individuals confirming infection, but only VI-positive in 16/20 individuals on at least 1 day leaving 4 individuals VI-negative across all testing days. The total dog screening results across those same 4/20 VN-positive but VI-negative cattle were 64.17% (±11.16) in sensitivity. These data appear to suggest that the presence of virus detectable by traditional means (i.e., VI) in a sample is not necessary for odor recognition and the metabolic processes that occur due to infection, non-intact virus and/or genetic material are more suggestive to result in the unique biological agent-based odor profile for BVDV distinct from BoHV-1. Use of culture-based training advancing to sample testing with successful generalization in dogs is also suggestive that a systemic response to infection is not necessary for presentation of a unique biological agent-based odor profile in BVDV.

A measure of quality assurance to monitor for possible contamination of training aid materials despite strict indirect charging conditions with clean technique was performed using the highest potential state of risk: a set of fresh unautoclaved POCRs for each virus used, BVDV and BoHV-1. The results of this surveillance showed rare, low-level contamination of genetic material present with no live virus detected on any samples. These results support the overall need for the rigorous sterilization process for safe processing and fielding of training aids to operational canine teams.

This study indicates a capability for safely training detection canines in the context of restricted and hazardous biological targets using the POCR training aid. Future studies should evaluate the best training methods for generalization from POCR to live animal and field-based testing. Using a controlled prospective study to evaluate windows of detection (early incubation period vs. non-infective recovery) beyond the peak infective period will be useful in establishing the limits of this capability. In addition, the analyses of the VOCs may reveal an odor fingerprint that is biological agent-specific which could be applied toward electronic sensing and screening modalities. The characteristics of the odors emitted by the biological targets in this study are unknown; therefore, extrapolating results with targets in this study to other biological targets should be done with caution. Any specific target biological agent needs to be tested in a manner that illustrates operational effectiveness. The odors that dogs use to interpret the viral cultures and which odors of the viral culture are captured and delivered by POCR are currently unknown. The practical utility of the detection dogs' capability demonstrated in this study should be further investigated through operational testing.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Auburn University College of Veterinary Medicine Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

CA, TP, and MS: conceptualization and methodology. TF, TP, and MS: data collection. SS, SK, LL, and MS: analysis. CA, SK, and MS: writing—original draft preparation. CA, LW, PW, and MS supervision. All authors contributed to review and editing and approved the final version of the manuscript.

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Conflict of Interest: CA, TP, TF, and LW are represented on the patent, pending (TRAINING AID Patent Pending 2020 338518: 47-19 US).

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Detection of Post-COVID-19 Patients Using Medical Scent Detection Dogs—A Pilot Study

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There is a growing number of COVID-19 patients experiencing long-term symptoms months after their acute SARS-CoV-2 infection. Previous research proved dogs' ability to detect acute SARS-CoV-2 infections, but has not yet shown if dogs also indicate samples of patients with post-COVID-19 condition (Long COVID). Nine dogs, previously trained to detect samples of acute COVID-19 patients, were confronted with samples of Long COVID patients in two testing scenarios. In test scenario I (samples of acute COVID-19 vs. Long COVID) dogs achieved a mean sensitivity (for acute COVID-19) of 86.7% (95%CI: 75.4–98.0%) and a specificity of 95.8% (95%CI: 92.5–99.0%). When dogs were confronted with Long COVID and negative control samples in scenario IIa, dogs achieved a mean sensitivity (for Long COVID) of 94.4 (95%CI: 70.5–100.0%) and a specificity of 96.1% (95%CI: 87.6–100.0%). In comparison, when acute SARS-CoV-2 positive samples and negative control samples were comparatively presented (scenario IIb), a mean sensitivity of 86.9 (95%CI: 55.7–100.0%) and a specificity of 88.1% (95%CI: 82.7–93.6%) was attained. This pilot study supports the hypothesis of volatile organic compounds (VOCs) being long-term present after the initial infection in post-COVID-19 patients. Detection dogs, trained with samples of acute COVID-19 patients, also identified samples of Long COVID patients with a high sensitivity when presented next to samples of healthy individuals. This data may be used for further studies evaluating the pathophysiology underlying Long COVID and the composition of specific VOC-patterns released by SARS-CoV-2 infected patients throughout the course of this complex disease.

Keywords: SARS-CoV-2, scent detection dogs, Long COVID, volatile organic compound (VOC), COVID-19

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INTRODUCTION

Due to their extraordinary olfaction capabilities and trainability dogs can be deployed not only for the detection of explosives, drugs, or missing persons but also for the identification of medical conditions including viral infections (1, 2). Since April 2020, we have been training and deploying dogs to detect samples from individuals with severe acute respiratory syndrome coronavirus 2

(SARS-CoV-2) infection, using different human body fluids, such as sweat, saliva and urine of infected patients (3, 4). With samples of acute coronavirus disease 2019 (COVID-19)-patients, several research groups have shown a detection sensitivity close to 95% and a specificity of 97% for confirmed cases (positive RT-PCR) vs. SARS-CoV-2-negative subjects (3–8). In addition, our group has shown that dogs can differentiate SARS-Co-V-2 infected material not only from control samples but also from samples of patients with other respiratory viral infections, including other coronaviruses (9). It is thought that the specific odor of an infection is composed of a unique pattern of volatile organic compounds (VOCs). The specific VOC pattern of a SARS-CoV-2 infected individual as well as of those with other viral infections is still under investigation (10). It is interesting to note that canines, in contrast to humans, have the Jacobson vomero-nasal organ (VNO), which is characterized by a different mechanism of odor perception, of which the main function is intra-species communication *via* the detection of pheromones, but it can also sense a wide variety of molecules (11). It has been speculated that VNO may detect viral proteins (11).

Whereas, most patients fully recover from COVID-19, a significant proportion experiences long-term-symptoms (12). A recent study found a prevalence of post-acute symptoms among people with COVID-19 in the UK between 3.0% (based on tracking specific symptoms) to 11.7% (based on self-classification) (13). The WHO recently published a clinical case definition of post-COVID-19 condition by a Delphi consensus (12). According to the WHO "post-COVID-19 condition occurs in individuals with a history of SARS-CoV-2 infection, usually 3 months from onset of COVID-19 with symptoms that last for at least 12 months and cannot be explained by an alternative diagnosis" (12). Common symptoms include fatigue, shortness of breath, muscle pain, cough, cognitive impairment, memory loss and sleep disorders, all leading to reduced quality of life of affected patients (14). In the following we will refer to post-COVID-19 condition as "Long COVID."

Up to now, the underlying mechanisms of Long COVID are not fully understood, and current studies are now gradually providing valid data to better understand this condition. One widely discussed hypothesis for the underlying cause of Long COVID is the persistence of viral RNA. The persistence of SARS-CoV-2-RNA has been described for olfactory slots (15), brain (16), whereas viral protein persistence has been detected in monocytes (17).

Therefore, it is of great scientific interest to assess whether COVID-19-detection-dogs, trained with samples of acutely SARS-CoV-2-infected patients, can identify samples of Long COVID patients as SARS-CoV-2-positive, as this would support the hypothesis of SARS-CoV-2-persistence or persistent metabolic alterations leading to characteristic VOC patterns in Long COVID patients.

MATERIALS AND METHODS

Long COVID patients were recruited at the Department of Respiratory Medicine at Hannover Medical School (MHH; ethic consent number 9042_BO_K_2020). All patients had an initial acute infection with SARS-CoV-2 (verified by RT-qPCR) and prolonged symptoms. Saliva samples (1–3 ml) were collected at the MHH and immediately deep-frozen at −80°C in the laboratory until usage. In addition to saliva samples of Long COVID-patients, negative saliva, urine and sweat from healthy individuals (SARS-CoV-2 RT-qPCR negative, with no previous history of COVID-19, nor a history of a recent cold or infection, recruited at multiple locations) as well as saliva, urine and sweat samples of acute COVID-19 patients (SARS-CoV-2-RT-qPCR positive, hospitalized as well as non-hospitalized) were included in the study as described in detail by Jendrny et al. (3, 4). Based on former results showing that beta-propiolactone (BPL) inactivation does not change scent dog detection, all samples of acute COVID-19 patients and Long COVID patients were inactivated with BPL according to the protocol described in Jendrny et al. to provide safe training conditions for dogs and handlers (3, 4). Characteristics of the recruited patients are summarized in **Supplementary Table 1**. A volume of 100 µl per sample was pipetted onto a cotton swab (for saliva and urine) or the cotton pad that was used to acquire the sweat sample itself was placed into a 4 ml glass tube and placed in a device called "Detection Dog Training System" (DDTS, Kynoscience UG, Hörstel, Germany) for training and testing as described in our previous studies (3, 4, 9). The DDTS allows for rapid, automatic, randomized, trainer-bias devoid and double-blind sample presentation (3, 4, 9). To verify the recorded results of the DDTS the dogs were filmed during testing and the videos were analyzed manually. In total, nine dogs (seven females and two males) were included. All dogs completed obedience training before the study, were all trained for detection of acute SARS-CoV-2-positive samples and participated in our former studies (3, 4, 9). For the present study the training period could be shortened to 3 days as all dogs were still able to distinguish positive and negative samples with high accuracies. We used saliva, urine and sweat samples of SARS-CoV-2-RT-qPCR positive patients (inactivated with BPL) and of SARS-CoV-2 RT-qPCR negative individuals for training. Samples used for training were never presented again to the dogs during the subsequent testing procedure, guaranteeing novelty of samples for validation purpose.

Two test scenarios were performed. For test scenario I, acute SARS-CoV-2 positive saliva samples and Long COVID saliva samples were presented to the dogs *via* DDTS. In test scenario II, acute SARS-CoV-2 positive (saliva, sweat and urine), Long COVID (saliva) as well as SARS-CoV-2-negative control samples (saliva, sweat and urine) samples were placed in the DDTS. In test scenario II, either dogs were confronted with a Long COVID sample next to SARS-CoV-2-negative control samples (test scenario IIa) or an acute SARS-CoV-2-positive sample was presented next to SARS-CoV-2-negative control samples (test scenario IIb).

Every nose dip into the DDTS' slots was evaluated with four possible options as described before (3, 4, 9). The DDTS changed the positions of the presented samples without letting the dog, dog handler nor other personnel present in the testing room know the new positions of negative or positive samples.

TABLE 1 | Diagnostic performance of the scent detection dogs in test scenario I (Acute COVID-19 vs. Long COVID).

Dog	Detection	SARS-CoV-2 disease status		Total number of presented samples	Diagnostic specificity (Sp)	Diagnostic sensitivity (Se)	Confidence interval (95% CI) Sp	Confidence interval (95% CI) Se	Positive predictive value (PPV)	Negative predictive value (NPV)	Confidence interval (95% CI) PPV	Confidence interval (95% CI) NPV	Accuracy	Fisher's exact test, *p*-value
		acute	long COVID											
Lotta	Yes	10	4	73	0.9344	0.833	0.8432–0.9742	0.5520–0.9704	0.7143	0.9661	0.4535–0.8828	0.8846–0.994	0.9178	<0.0001
	No	2	57											
Baila	Yes	8	3	78	0.9545	0.6667	0.8747–0.9876	0.3906–0.8619	0.7273	0.9403	0.4344–0.9025	0.8563–0.9765	0.9103	<0.0001
	No	4	63											
Füge	Yes	10	6	61	0.8824	1	0.7662–0.9449	0.7225–1	0.625	1	0.3864–0.8152	0.9213–1	0.9016	<0.0001
	No	0	45											
Joe	Yes	10	0	86	1	0.8333	0.9507–1	0.5520–0.9704	1	0.9737	0.7225–1	0.9090–0.9953	0.9767	<0.0001
	No	2	74											
Vine	Yes	10	2	50	0.9487	0.9091	0.8311–0.9909	0.6226–0.9953	0.8333	0.9737	0.5520–0.9704	0.8651–0.9987	0.9400	<0.0001
	No	1	37											
Bella	Yes	10	0	68	1	1	0.9379–1	0.7225–1	1	1	0.7225–1	0.9379–1	1	<0.0001
	No	0	58											
Filou	Yes	10	2	58	0.9583	1	0.8602–0.9926	0.7225–1	0.8333	1	0.5520–0.9704	0.9229–1	0.9655	<0.0001
	No	0	46											
Erec	Yes	9	1	70	0.9825	0.6923	0.9071–0.9991	0.4237–0.8732	0.9	0.9333	0.5958–0.9949	0.8407–0.9738	0.9286	<0.0001
	No	4	56											
					Mean Sp	**Mean Se**	**95% CI of mean Sp**	**95% CI of mean Se**	**Mean PPV**	**Mean NPV**	**95% CI of mean PPV**	**95% CI of mean NPV**	**Mean accuracy**	**95% CI of mean accuracy**
					0.9576	**0.8668**	**0.9252-0.99**	**0.7539-0.9798**	**0.8292**	**0.9734**	**0.7158–0.9425**	**0.9513–0.9955**	**0.9426**	**0.9134–0.9717**

This allowed a double-blind sample presentation, controlled and recorded only by the DDTS' software and additional confirmation videos. In addition, all staff involved was positioned accordingly to prevent any interaction or influencing of the animals during the study.

Sample size and sample acquisition were conducted based on and according to our former studies (3, 4, 9). The diagnostic sensitivity as well as diagnostic specificity, positive predictive values (PPV), and negative predictive values (NPV) were calculated according to Trevethan (18). Ninety-five percent confidence intervals (CIs) for sensitivity, specificity, PPV, and NPV were calculated with the hybrid Wilson/Brown method (19). Means of sensitivity, specificity, PPV, NPV, and accuracy with corresponding 95% CIs of mean were also calculated per session. Two-tailed Fisher's exact test was used for analysis of the individual contingency tables; a $P \leq 0.05$ was considered significant. All calculations were done with the Prism 9 software from GraphPad (La Jolla, CA, USA).

The study was carried out in accordance with the ethical requirements established by the Declaration of Helsinki and was approved by the local Ethics Committee of MHH (ethic consent number 9042_BO_K_2020). Written informed consent from all participants was obtained before sample collection. Animal work according to the study protocol and design was approved by the German Armed Forces.

RESULTS

Overall, a total of 732 sample presentations were performed (**Tables 1**–**3**). When presenting acute COVID-19 samples and Long COVID samples (test scenario I), dogs made 436 rejections and only 18 indications of Long COVID samples (96.04 vs. 3.96%), while 77 correct indications and only 13 false rejections of acute COVID-19 samples were recorded. When presenting Long COVID samples next to SARS-CoV-2 negative samples (test scenario IIa), dogs only rejected a Long COVID sample once, while they indicated 13 Long COVID samples (7.14 vs. 92.86%). During this sample presentation in test scenario IIa, 47 correct rejections and only 2 false indications of SARS-CoV-2-negative samples were performed. During the presentation of acute COVID-19 vs. SARS-CoV-2-negative samples (test scenario IIb), 16 correct indications and 3 false rejections of acute COVID-19 samples were recorded, while 93 correct rejections and only 13 false indications of SARS-CoV-2 negative samples were made.

As shown in **Figure 1** dogs achieved a mean sensitivity of 86.7% (95%CI: 75.4–98.0%) and a specificity of 95.8% (95%CI: 92.5–99.0%) in test scenario I, where samples of acute COVID-19 vs. Long COVID were presented (**Table 1**). When dogs were confronted with Long COVID and negative control samples in scenario IIa, dogs achieved a mean sensitivity (for Long COVID) of 94.4% (95%CI: 70.5–100.0%) and a specificity of 96.1% (95%CI: 87.6–100.0%) (**Table 2**). In test scenario IIb, when acute SARS-CoV-2 positive samples and negative control samples were comparatively presented to the dogs, a mean sensitivity (for acute

TABLE 2 | Diagnostic performance of the scent detection dogs in test scenario IIa (Long COVID vs. negative controls).

Dog	Detection	SARS-CoV-2 disease status		Total number of presented samples	Diagnostic specificity (Sp)	Diagnostic sensitivity (Se)	Confidence interval (95% CI) Sp	Confidence interval (95% CI) Se	Positive predictive value (PPV)	Negative predictive value (NPV)	Confidence interval (95% CI) PPV	Confidence interval (95% CI) NPV	Accuracy	Fisher's exact test *p*-value
		Long COVID	negative											
Bella	Yes	4	1	21	0.941	1.000	0.730–0.997	0.510–1.000	0.800	1.000	0.376–0.990	0.806–1.000	0.952	0.0008
	No	0	16											
Margo	Yes	5	1	23	0.941	0.833	0.730–0.997	0.437–0.992	0.833	0.941	0.437–0.992	0.730–0.997	0.913	0.001
	No	1	16											
Erec	Yes	4	0	19	1.000	1.000	0.796–1.000	0.510–1.000	1.000	1.000	0.510–1.000	0.796–1.000	1.000	0.0003
	No	0	15											
					Mean Sp	Mean Se	95% CI of mean Sp	95% CI of mean Se	Mean PPV	Mean NPV	95% CI of mean PPV	95% CI of mean NPV	Mean accuracy	95% CI of mean accuracy
					0.961	0.944	0.876–1.000	0.705–1.000	0.878	0.980	0.612–1.000	0.896–1.000	0.955	0.847–1.000

TABLE 3 | Diagnostic performance of the scent detection dogs in test scenario IIb (Acute COVID-19 vs. negative controls).

Dog	Detection	SARS-CoV-2 infection status		Total number of presented samples	Diagnostic specificity (Sp)	Diagnostic sensitivity (Se)	Confidence interval (95% CI) Sp	Confidence interval (95% CI) Se	Positive predictive value (PPV)	Negative predictive value (NPV)	Confidence interval (95% CI) PPV	Confidence interval (95% CI) NPV	Accuracy	Fisher's exact test *p* value
		positive	negative											
Bella	Yes	6	5	52	0.886	0.750	0.760–0.951	0.409–0.956	0.546	0.951	0.280–0.787	0.839–0.991	0.865	0.0005
	No	2	39											
Margo	Yes	4	2	24	0.900	1.000	0.699–0.982	0.510–1.000	0.667	1.000	0.300–0.941	0.824–1.000	0.917	0.0014
	No	0	18											
Erec	Yes	6	6	49	0.857	0.857	0.722–0.933	0.487–0.993	0.500	0.973	0.254–0.746	0.862–0.999	0.857	0.0004
	No	1	36											
					Mean Sp	**Mean Se**	**95% CI of mean Sp**	**95% CI of mean Se**	**Mean PPV**	**Mean NPV**	**95% CI of mean PPV**	**95% CI of mean NPV**	**Mean accuracy**	**95% CI of mean accuracy**
					0.881	**0.869**	**0.827–0.936**	**0.557–1.000**	**0.571**	**0.975**	**0.357–0.785**	**0.914–1.000**	**0.880**	**0.800–0.960**

COVID-19) of 86.9% (95%CI: 55.7–100.0%) and a specificity of 88.1% (95%CI: 82.7–93.6%) could be attained (**Table 3**).

DISCUSSION

Several studies have shown that dogs can be trained to distinguish samples of acutely SARS-CoV-2-infected patients from samples of SARS-CoV-2-negative, healthy controls as well as from other viral infections with high diagnostic sensitivity and specificity (3–9). In the current study, trained SARS-CoV-2-detection dogs were confronted with samples of Long COVID patients for the first time. During their training period only samples of acutely SARS-CoV-2-infected patients were used as target scent.

92.86% of Long COVID samples were indicated as SARS-CoV-2-positive, when Long COVID samples were presented next to SARS-CoV-2-negative control samples. Interestingly, when Long COVID samples were presented next to acute SARS-CoV-2-positive samples, dogs only indicated 3.96% of Long COVID samples as positive. These results suggest that the disease-specific odor of acute COVID-19 is still present in the majority of Long COVID samples, but probably not to the same extent as in samples of acutely infected COVID-19 patients. In other words, when acute COVID-19 samples are presented next to Long COVID samples the dogs rather indicate the samples from acute cases, with the smell they were trained on. In a recently published study performed by Grandjean et al., dogs identified only 51.5% of Long COVID patients when they were presented next to healthy individuals (20). The lower percentage of identified Long COVID patients compared to our results (51.5 vs. 92.86%) might be explained by the differing sample quality as the samples used by Grandjean et al. were taken at home and were sent *via* mail without standardized freezing or cooling of the samples (20). Nevertheless, these results also support the hypothesis that the disease-specific odor of acute COVID-19 is still present in the majority of Long COVID samples, but probably not as strong as in samples of acutely infected COVID-19 patients (20).

The disease-specific odor that can be detected by dogs is thought to be determined by a specific pattern of VOCs. VOCs are produced by cell metabolism and released with breath, urine, saliva, blood, sweat and other body fluids (21). As viruses have no metabolism, the common hypothesis is that viruses change the metabolism of the infected host and therefore generate a special VOC pattern (22). The nature of these VOCs is currently being identified by several international laboratories in different countries and data suggest that SARS-CoV-2 infections create a specific VOC pattern (23–25).

Apart from detecting VOC patterns, dogs might also be able to directly detect viral proteins with their vomeronasal organ (VNO). The VNO can process a wide range of molecules, including proteins (26, 27). This fact and the results generated in the present study support data on persistence of SARS-CoV-2 as documented in the literature for post-COVID-19 condition patients (15–17). Up to date, it had not been demonstrated whether it corresponds to the replicative virus or not. The

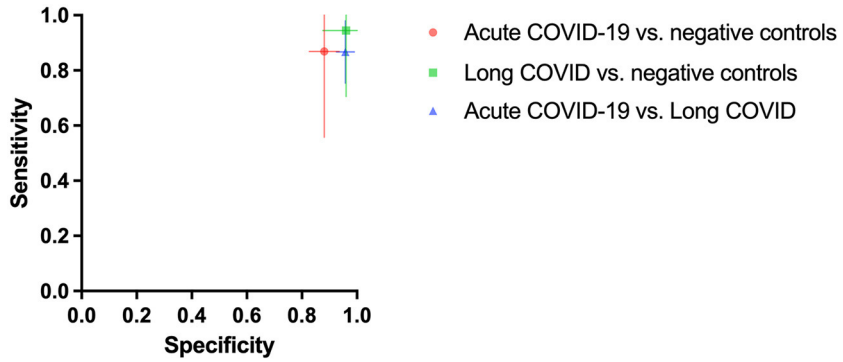

FIGURE 1 | Mean diagnostic specificity and sensitivity for all dogs for acute COVID-19 vs. negative control (red circle), Long COVID vs. negative control (green square), and acute COVID-19 vs. Long COVID (blue triangle) samples, respectively. The 95% confidence intervals of the means for specificity and sensitivity are shown with horizontal and vertical bars, respectively.

canine detection test supports the hypothesis that the virus still replicates at least to a limited extent, after the acute phase of COVID-19. It may be possible that this occurs in various body regions such as olfactory mucosa (15), brain (16), and in monocytes (17), even if a nasopharyngeal swab PCR has become negative.

The results of the current study could suggest the hypothesis of SARS-CoV-2 persistence in Long COVID patients months after their acute SARS-CoV-2 infection, but the study should be regarded as a pilot study due to inclusion of a limited number of patients. Further research with more patients and samples acquired from the same patient at different time points is needed, to evaluate to what extent the sensitivity of medical detection dogs may vary throughout the course of the infection. For a better understanding of the pathophysiology of post-COVID-19 condition, future studies with higher sample sizes should also address the questions if the nature of the symptoms influences the detection performance of the dogs, as there has been a variety of symptoms described for post-COVID-19 condition. Furthermore, studies characterizing disease specific VOCs, should generate a deeper understanding of what scent detection dogs detect in SARS-CoV-2 infected individuals.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Hannover Medical School (consent number 9042_BO_K_2020). The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by German Armed Forces.

AUTHOR CONTRIBUTIONS

FT was responsible for the planning of the study, conducted and coordinated the sample acquisition, carried out data analyses, and drafted the manuscript. NtH, SM, and HV designed and coordinated the study, carried out the main practical work (NtH and PJ) and were responsible for data analyses (SM). AO and CS participated in the planning of the laboratory part of the study and were in charge for the legal permission for sample processing. CS carried out the laboratory work at the Research Center for Emerging Infections and Zoonoses including sample preparation, virus inactivation, and RT-qPCR. HE programmed the DDTS software and supported the dog training. IP, ND, and TW were in charge for the ethical approval, patient recruitment, and sample collection at Hannover Medical School. ES was involved in designing and coordinating the study and was responsible for the dog training and helped with data analyses. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.877259/full#supplementary-material

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Conflict of Interest: HE was employed by KynoScience UG.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Use of trained scent dogs for detection of COVID-19 and evidence of cost-saving

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Background: One of the lessons learned from the coronavirus disease 2019 (COVID-19) pandemic is the importance of early, flexible, and rapidly deployable disease detection methods. Currently, diagnosis of COVID-19 requires the collection of oro/nasopharyngal swabs, nasal turbinate, anterior nares and saliva but as the pandemic continues, disease detection methods that can identify infected individuals earlier and more quickly will be crucial for slowing the spread of the virus. Previous studies have indicated that dogs can be trained to identify volatile organic compounds (VOCs) produced during respiratory infections. We sought to determine whether this approach could be applied for detection of COVID-19 in Rwanda and measured its cost-saving.

Methods: Over a period of 5 months, four dogs were trained to detect VOCs in sweat samples collected from human subjects confirmed positive or negative for COVID-19 by reverse transcription polymerase chain reaction (RT-PCR) testing. Dogs were trained using a detection dog training system (DDTS) and *in vivo* diagnosis. Samples were collected from 5,253 participants using a cotton pad swiped in the underarm to collect sweat samples. Statistical analysis was conducted using R statistical software.

Findings: From August to September 2021 during the Delta wave, the sensitivity of the dogs' COVID-19 detection ranged from 75.0 to 89.9% for the lowest- and highest-performing dogs, respectively. Specificity ranged from 96.1 to 98.4%, respectively. In the second phase coinciding with the Omicron wave (January–March 2022), the sensitivity decreased substantially from 36.6 to 41.5%, while specificity remained above 95% for all four dogs. The sensitivity and specificity by any positive sample detected by at least one dog was 83.9, 95% CI: 75.8–90.2 and 94.9%; 95% CI: 93.9–95.8, respectively. The use of scent detection dogs was also found to be cost-saving compared to antigen rapid diagnostic tests, based on a marginal cost of approximately $14,000 USD for testing of the 5,253 samples which makes 2.67 USD per sample. Testing turnaround time was also faster with the scent detection dogs, at 3 h compared to 11 h with routine diagnostic testing.

Conclusion: The findings from this study indicate that trained dogs can accurately identify respiratory secretion samples from asymptomatic and symptomatic COVID-19 patients timely and cost-effectively. Our findings recommend further uptake of this approach for COVID-19 detection.

KEYWORDS

COVID-19, SARS-CoV-2, volatile organic compounds (VOCs), scent dogs, RT-PCR, cost-saving

Introduction

Since its recognition as a public health emergency of international concern in January 2020, the coronavirus disease 2019 (COVID-19) has spread around the world, and was declared a pandemic by the World Health Organization (WHO) in March, 2020 (1). Effective management of infectious diseases depends on reliable and timely diagnosis (2) and in the case of COVID-19, the gold standard diagnostic test is the Reverse Transcription-Polymerase Chain Reaction (RT-PCR) test using oro/nasopharyngeal swabs or other upper respiratory tract specimens. Unfortunately, this method of testing is not widely available in low- and middle-income countries (LMICs) due to the lack of reagents' supply and low testing capacity (3). Furthermore, RT-PCR tests can be time-consuming to process, and can produce false positive or negative results (3). These limitations have led to significant challenges in LMICs. In early 2020, the Government of Rwanda built on existing RT-PCR testing capabilities acquired during the Ebola Virus Disease (EVD) epidemic to improve early detection of COVID-19 (Rwanda COVID-19 Intra Action Review, 2020). The ability to detect COVID-19 (either using RT-PCR or rapid antigen tests) was rapidly extended to all healthcare facilities in the country. However, there were challenges due to the complexity of RT-PCR testing, and although new innovative testing strategies were developed, these approaches still required extensive laboratory equipment and trained laboratory experts (4). These challenges resulted in delays of both case detection and management. While the recent introduction of rapid antigen tests has significantly reduced the turnaround, time needed to provide patients with results, there is still a need for faster and easier ways of detecting COVID-19 to enable appropriate and cost effective COVID-19 test.

One approach to the rapid detection of COVID-19 is through the use of medical scent detection dogs, which can rapidly detect volatile organic compounds (VOCs) associated with coronavirus with a high degree of specificity, sensitivity, and accuracy for a large number of individuals (5, 6). Evidence of dogs' efficacy in detecting medical conditions and diseases (either communicable or non-communicable) has been reported in studies conducted in Germany and UK (7, 8). In Germany for example, a study conducted with eight detection dogs on 1,012 randomized samples resulted in an overall detection rate of 94%, while sensitivity and specificity rates were 82.63 and 96.35%, respectively (9). Several studies have shown the ability of medical scent detection dogs to identify samples from SARS-CoV-2 infected individuals with high accuracy, highlighting the role such dogs could play in the management of a pandemic (10–13). Previous research showed that different body fluids, such as saliva, sweat and urine and other sample types like worn face masks are suitable for detection, which suggests that there is a general SARS-CoV-2 infection associated odor that dogs can be trained on (13, 14). In addition, our group demonstrated that

such dogs were able to differentiate SARS-CoV-2 infection from other acute viral respiratory tract infections (7). However, most of the current data were generated in laboratory settings, rather than in a real-world scenario.

Our study sought to test the concept of using dogs to reliably differentiate between samples from patients infected with COVID-19 and non-infected controls in Rwanda. To our knowledge, this is the first study of its kind to be conducted in a LMIC.

Materials and methods

Study design and setting

This study was a cross-sectional design to assess the validity of the scent dog test for COVID-19 using sweat samples from both symptomatic and asymptomatic patients. Between March and July 2021, we performed trainings of dogs and handlers in regard to the sensitivity and specificity compared to RT-PCR gold standard's results, followed by a pilot using 61 known samples. These sweat samples and oro-nasal pharyngeal swabs were collected from symptomatic and asymptomatic individuals as described below. In this pilot phase, dogs learned to identify COVID-19 sweat samples directly by smelling the human body odors present in a cotton pad that participants swiped in their armpit. After this pilot phase, in August 2021 we initiated the first validation phase where four dogs were continuously trained to detect COVID-19 in sweat samples collected from both symptomatic and asymptomatic individuals admitted at King Faisal Hospital and the University Teaching Hospital of Kigali (CHUK), respectively. In addition, we also collected samples from the participants recruited across the country in high spot areas from the City of Kigali, Western, Southern, Northern, and Eastern provinces of Rwanda. Samples were also collected from markets, bars, restaurant, and churches during random drive through national outreach COVID-19 testing campaigns. This phase coincided with the wave associated with the surge of Delta variant which took place between July and mid-December 2021 in Rwanda.

In the second validation phase corresponding to the wave of Omicron variant which started late December 2021, we continued to collect and process the same samples until March. There was no incentive involved in the recruitment and sample collection process. All tests were performed free of charge as part of national response to COVID-19 in the interest of public health.

Sample size

In total, 5,253 sweat samples (in addition to 61 samples collected during the pilot) were collected from symptomatic, asymptomatic and non-infected individuals for COVID-19 patients aged 18 years and above from August 2021 to March 2022 covering two periods of Delta and Omicron variants' waves.

Specimen collection

Two types of samples were collected from consented both symptomatic patients and non-infected individuals upon their arrival at the hospital or site of sample collection. The first sample type was an oro-pharyngeal swab collected from the tonsils and posterior pharynx wall. Swab heads were immersed in 3 ml Viral Transport Medium (VTM), following manufacturer's guidelines, and then sent directly to the National Reference Laboratory (NRL/RBC) for RT-PCR testing. The second sample type was a self-collected sweat sample from all symptomatic and asymptomatic patients. Each patient was briefed on proper sample self-collection, which comprised of swiping a cotton pad (Wattenschijfjes Disque à Démaquiller, Everyday) in both armpits for at least 5 min and placing it into a glass jar. Samples were stored in the laboratory between 4–8°C until the time of testing, and at −80°C for long term bio-banking. In addition, we also collected saliva samples for bio-banking for further studies.

Reverse transcription polymerase chain reaction testing

Reverse transcription polymerase chain reaction was considered the gold standard test against which to compare the scent detection dogs' performance. All dog handlers were fully equipped with proper personal protective equipment (PPE) every time they were handling dogs or/and samples. Oropharyngeal RNA samples were extracted with a DAAN RNA/DNA Purification Kit (8). A total of 5 μl of extracted RNA were added to 20 μl of a master mix to make a solution of 25 μl, as per manufacturer's guidelines. The RT-PCR test for detection of SARS-CoV-2 was done using 2019-nCoV RNA RT-PCR kit targeting two genes [orf1ab1ab known as open reading frame and nucleocapsid protein (*N*)] as described by manufacturer (DAAN Gene Co., Ltd., Of Sun Yat-sen University, 19, Xiangshan Road, Guangzhou Hi-Tech Industrial Development Zone, China). The solution was run on the Bio-Rad CFX96 thermocycler at 50°C for 15 min for reverse transcription, denatured at 95°C for 15 min, followed by 45 PCR cycles at 94°C for 15 s and 55°C for 45 s. The average turnaround time for RT-PCR was 21/2 h. A cycle threshold value (*Ct*) of more than or equal to 37 indicated a negative test result. Positive controls for the reaction showed amplification as determined by curves for FAM and VIC detection channels (4).

Sniffer dogs' characteristics

This study was conducted in collaboration with the canine department of the Rwanda National Police at Kigali International Airport. The dogs were supplied by Police Dogs Centre Holland BV, RJ Sint-Oedenrode, The Netherlands. They were selected according to features such as age, breed, and sex. The dogs' characteristics are displayed in Table 1.

Detection dog training system

The Detection Dog Training System (DDTS, Kynoscience UG, Germany) was used for training dogs. The system is composed of seven "sniffing holes" attached to tubes (Figure 1). Behind each hole there is a tube leading to a metal container. Each metal container is covered with a grid, which allows the odor to escape and reach the sniffing hole. Each tube L-shaped order to prevent physical contact with the samples and to avoid any visual cues that may impact results.

Scent dog detection facility set up and use of olfactory cones

The scent dog detection facility was set-up at Kigali International Airport with objective to scale-up this testing strategy in collaboration with Canine brigade of Rwanda National Police and Rwanda Airports Company and for strengthening infection prevention and control (IPC) measures against COVID-19 in the country with limited cost. This facility was made up of three rooms including the testing room, the DDTS room, and a staff room (Figure 2).

As the DDTS machine has a limited throughput related to logistics, custom olfactory cone products were developed for the actual specimen testing and for easy scale-up locally. These olfactory cones were locally made from a funnel to be used by the dogs during the detection of VOCs. The funnels were attached to a bottle containing a cotton pad used to collect sweat samples (Figure 3).

Training of handlers and scent dogs for detection of COVID-19

Dogs' handlers received a pilot training in basic commands, dogs' learning behavior, and different rewarding methods. The four dogs were first trained for detection of COVID-19 using DDTS. The dogs were introduced to the sweat samples of patients with COVID-19 and healthy controls so that they become familiar with these secretions. Sweat samples stored in appropriate storage temperature as described in specimen collection method. After being collected samples were transported every day from the National Reference Laboratory to the training site at Kigali International Airport. The samples were then placed in the olfactory cones and each dog smelled the secretion in each cone in order to learn how to distinguish positive from negative samples. Each dog smelled each sample for around 1 s and then moved to the next one. When the dog indicated a positive sample, the dog stopped at the olfaction cone for 3–4 s. The dog indication behavior attracted attention of the handlers, and the dog was rewarded and then continued to next cones.

After intensive training, each dog could smell an average of 50 samples within 3 min. After each day's training, samples were re-stored in Kigali International Airport Molecular Laboratory.

Safety measures

Before starting the study, the dogs' handlers were tested for COVID-19 using RT-PCR testing. The handlers were also familiar about COVID-19 symptoms and how to respond to a potential exposure. They were then re-tested regularly every 2 weeks over the course of the pilot study. COVID-19 prevention measures were taken to prevent infection throughout the study, including the use of PPE (i.e., face masks, face shields, and lab coats). All samples were transported in accordance with recommended procedures. The dogs were kept in standard crates in accordance with ethical guidelines, and were fed high-quality dog food throughout the study by veterinary doctors.

Statistical analysis

We disaggregated our analysis into two time periods that correspond with waves of differing dominant COVID-19 variants in the country during the study period. The first period was from August to September 2021, when the Delta variant was dominant, while the second period was from January to March 2022, when the Omicron variant was dominant. The period from October to December 2021 was removed from the analysis as there was no positive case identified by the PCR test even if scent dogs continued testing. We combined results from all dogs to generate a new binary variable (1: positive with at least one dog and 0 for negative to all dogs). This categorization was based on probable impact of variant to the performance

TABLE 1 Dogs' characteristics.

Name	Age (year)	Sex	Breed
Dog 1	2	Male	Labrador
Dog 2	2	Male	Labrador
Dog 3	2	Male	Malinois
Dog 4	2	Female	Malinois

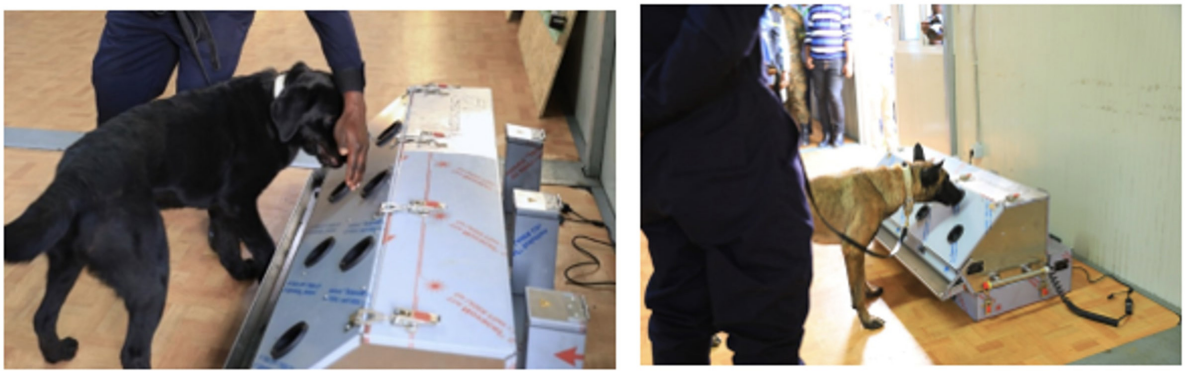

FIGURE 1
The Detection Dog Training System (DDTS, Kynoscience UG, Germany) used for training dogs. This machine system has seven sniffing holes attached to tubes where scent dogs detect COVID-19 samples.

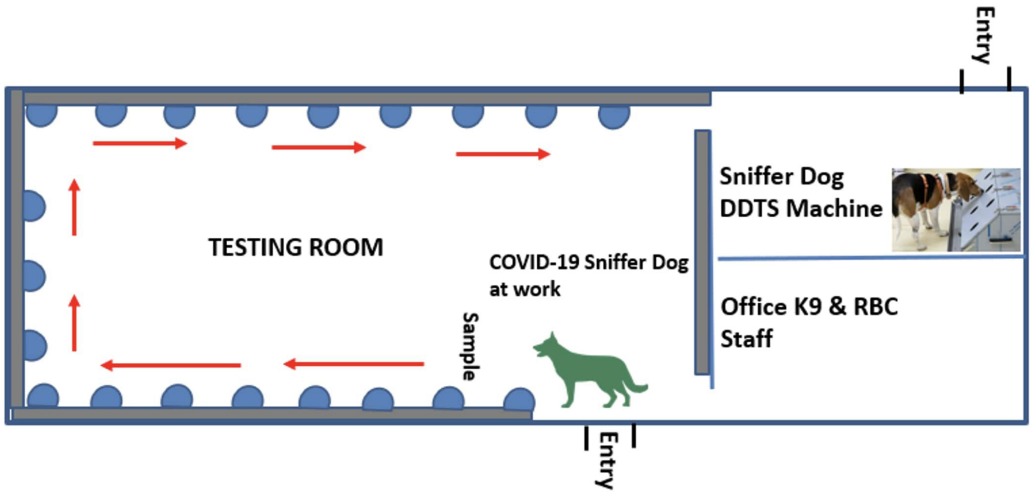

FIGURE 2
A scent dog detection facility constructed at the Kigali International Airport comprising of three rooms including the testing room, the DDTS room, and a staff room.

of scent dogs. Sensitivity, specificity, positive predictive value, and negative predictive value as well as the Receiver Operating Characteristic (ROC) were calculated in comparison with the RT-PCR results, considered as gold standard. In addition, the agreement level and a Kappa coefficient (k) was calculated to measure the level of agreement between scent detection dogs and RT-PCR testing.

Results

In our analysis of detection dogs' diagnostic performance with sweat samples, a total of 5,253 sweat samples were collected from major hospitals, treatment centers, markets, churches, and other hot spot areas across the country during the peak of the Delta and Omicron variants. Overall, 4.0% (123/3,071) of individuals tested positive for COVID-19 using RT-PCR. Results show a high positive yield of 12.4% (84/678) in period-1 (August to September 2021) and 1.63% (23/2.393) in period-2 (January to March 2022) ($P < 0.05$). Similarly, the positive yield using sniffer dogs ranged from 11.8 to 13.7% in period 1 and from 2.4 to 3.9% in period-2. The Kappa coefficient varied from 0,7 to 0,9 in the period-1 indicating a substantial agreement. However, results showed that the kappa coefficient was reduced to 0.3 and 0.2 in the period-2, showing a fair agreement (Table 2).

From August to September 2021 while we were in the period of Delta wave, the sensitivity of the dogs' COVID-19 detection ranged from 75.0 to 89.9% for the lowest- and highest-performing dogs, respectively. Specificity ranged from 96.1 to 98.4%, respectively. In the second period coinciding with the

FIGURE 3
Locally manufactured olfaction cones used by dogs for detection of COVID-19 in sweat samples collected from individuals on a cotton pad carried in a glass container. The containers are covered with grids, which allowed the odor to escape and reach the sniffing hole. Each tube extension is identical and shaped in a way that it prevents dogs from physical contact with the samples.

TABLE 2 Number of sniffed sweat samples per dog and level of agreement with RT-PCR.

Dog	Period	Number of sniffed sweat samples	Tested positive	Agreement with RT-PCR test %	Kappa (k)*
			n (%)	(95% CI)	
Dog 1	Total	3,071	144 (4.7%)	98.0 (97.3; 98.8)	0.6
	August–September 2021	678	80 (11.8%)	96.5 (95.5; 97.5)	0.8
	January–March 2022	2,393	64 (2.7%)	96.9 (95.9; 97.8)	0.3
Dog 2	Total	3,057	166 (5.4%)	97.9 (97.1; 98.6)	0.6
	August–September 2021	664	89 (13.4%)	96.8 (95.8; 97.8)	0.9
	January–March 2022	2,393	77 (3.2%)	96.5 (95.6; 97.4)	0.3
Dog 3	Total	2,842	144 (5.1%)	97.9 (97.2; 98.7)	0.6
	August–September 2021	678	93 (13.7%)	94.3 (93.3; 95.3)	0.7
	January–March 2022	2,164	51 (2.4%)	97.3 (96.3; 98.2)	0.3
Dog 4	Total	2,497	153 (6.1%)	96.5 (95.6; 97.3)	0.6
	August–September 2021	664	80 (12.1%)	94.4 (93.4; 95.4)	0.7
	January–March 2022	1,833	73 (3.9%)	95.6 (94.6; 96.5)	0.2

*Kappa coefficient (k) helps to measure the level of agreement produced during the detection of SARS-CoV-2 between scent dogs and RT-PCR.

Omicron wave (January–March 2022), the sensitivity decreased substantially ranging from 36.6 to 41.5%, while specificity remained above 95% for all four dogs (Table 3). The sensitivity and specificity by any positive detected by at least one dog were 83.9, 95% CI: 75.8–90.2 and 94.9%; 95% CI: 93.9–95.8, respectively.

The period of delta variant was characterized by low orf1ab and *N* genes C*t*-values, severe symptoms, many deaths and high viral load while omicron variant period was marked by high C*t*-values, mild symptoms, low viral load, and very few deaths (Figure 4). This is scientific evidence regarding impact of SARS-CoV-2 variants vs. sniffer dogs' performance. It is worth noting that mean average C*t*-values for RT-PCR SARS-CoV-2 detection rate was 31 and 36 during Delta and Omicron waves, respectively (Figure 4).

Cost minimization analysis and turnaround time

We also considered the cost effectiveness of using scent dogs for detection of COVID-19 compared to rapid antigen

TABLE 3 The detection dogs' performance.

	Overall		Period 1 (August–September 2021)		Period 2 (Jan–March 2022)	
	Percent	95% CI	Percent	95% CI	Percent	95% CI
Dog 1						
Sensitivity	68.5	(59.7–76.3)	83.1	(73.7–90.2)	36.6	(22.1–53.1)
Specificity	98.0	(97.4–98.4)	98.4	(97.1–99.2)	97.9	(97.2–98.4)
ROC area	83.2	(79.2–87.2)	90.8	(87.0–95.0)	67.2	(59.8–74.7)
Positive predictive value	59.3	(51.0–67.3)	88.1	(79.2–94.1)	21.2	(11.1–34.7)
Negative predictive value	98.6	(98.1–99.0)	97.6	(96.1–98.7)	99.1	(98.6–99.3)
Dog 2						
Sensitivity	70.6	(61.5–78.6)	82	(72.5–89.4)	36.7	(19.9–56.1)
Specificity	97.7	(97.0–98.2)	96.1	(94.3–97.5)	98.1	(97.4–98.6)
ROC area	84.1	(80.0–88.2)	89.1	(85.0–93.0)	67.4	(58.6–76.2)
Positive predictive value	56.4	(48.0–64.5)	75.3	(65.5–83.5)	41.9	(29.1–55.7)
Negative predictive value	98.7	(98.2–99.1)	97.4	(95.8–98.5)	97.6	(96.9–98.2)
Dog 3						
Sensitivity	74.6	(66.2–81.8)	89.9	(81.7–95.3)	41.5	(26.3–57.9)
Specificity	97.4	(96.7–97.9)	97.4	(95.7–98.5)	97.4	(96.6–98.0)
ROC area	86.0	(82.2–89.8)	93.6	(90.0–97.0)	69.0	(61.8–77.0)
Positive predictive value	55.1	(47.4–62.6)	83.0	(74.4–90.2)	21.3	(12.9–31.8)
Negative predictive value	98.9	(98.4–99.2)	98.5	(97.2–99.3)	99.0	(98.5–99.3)
Dog 4						
Sensitivity	64.1	(55.1–72.3)	75.0	(64.6–83.6)	40.0	(24.9–56.7)
Specificity	96.9	(96.1–97.5)	97.2	(95.6–98.4)	96.7	(95.8–97.5)
ROC area	80.5	(76.0–85.0)	86.1	(82.0–91.0)	68.4	(60.7–76.1)
Positive predictive value	51.9	(43.8–59.9)	79.5	(69.2–87.6)	21.3	(12.7–32.3)
Negative predictive value	98.1	(97.4–98.6)	96.4	(94.6–97.7)	98.7	(98.0–99.1)
At least one dog						
Sensitivity	83.9	(75.8–90.2)	97.6	(91.6–99.7)	44.8	(26.4–64.3)
Specificity	94.9	(93.9–95.8)	92.6	(90.1–94.6)	95.7	(94.6–96.7)
ROC area	90.0	(86.0–92.9)	95.1	(93.1–97.1)	70.3	(61.1–79.5)
Positive predictive value	46.3	(39.3–53.4)	65.9	(56.8–74.2)	16.3	(8.95–26.2)
Negative predictive value	99.1	(98.6–99.5)	99.6	(98.6–100.0)	99.0	(98.3–99.4)

test. While the cost of mass testing for COVID-19 using dogs is relatively constant over the number of screened persons, the cost of using rapid antigen tests increases with the number of tests performed. The estimated daily average cost of scent detection dogs was $79 USD, which is approximately equivalent to the cost of 24 rapid tests. The use of scent detection dogs was found to be cost-saving compared to Antigen rapid diagnostic tests, based on a marginal cost of approximately $14,000 USD for testing of the 5,253 samples which makes 2.67 USD per sample. When testing more than 24 samples, the use of dogs could minimize the cost of testing (**Figure 5**).

For estimating TAT, we calculated unit time in minute for testing using both RDT and scent detection dog (**Table 4**). Different variables including testing preparation, sample collection, sample transportation, sample processing, results and recording times have been considered for demonstrating TAT corresponding to each testing method. Overall results showed that the use of scent detection dog for testing one sample was 6.7 min per sample, while the use of RDT had an average TAT of 12.13 min per sample.

Discussion

This study demonstrated that the use of trained dogs for the detection of COVID-19 is a viable mass screening diagnostic approach with evidence of cost-savings. The use of scent detection dogs to detect diseases is not new in medical history. Different studies have demonstrated the ability of dogs

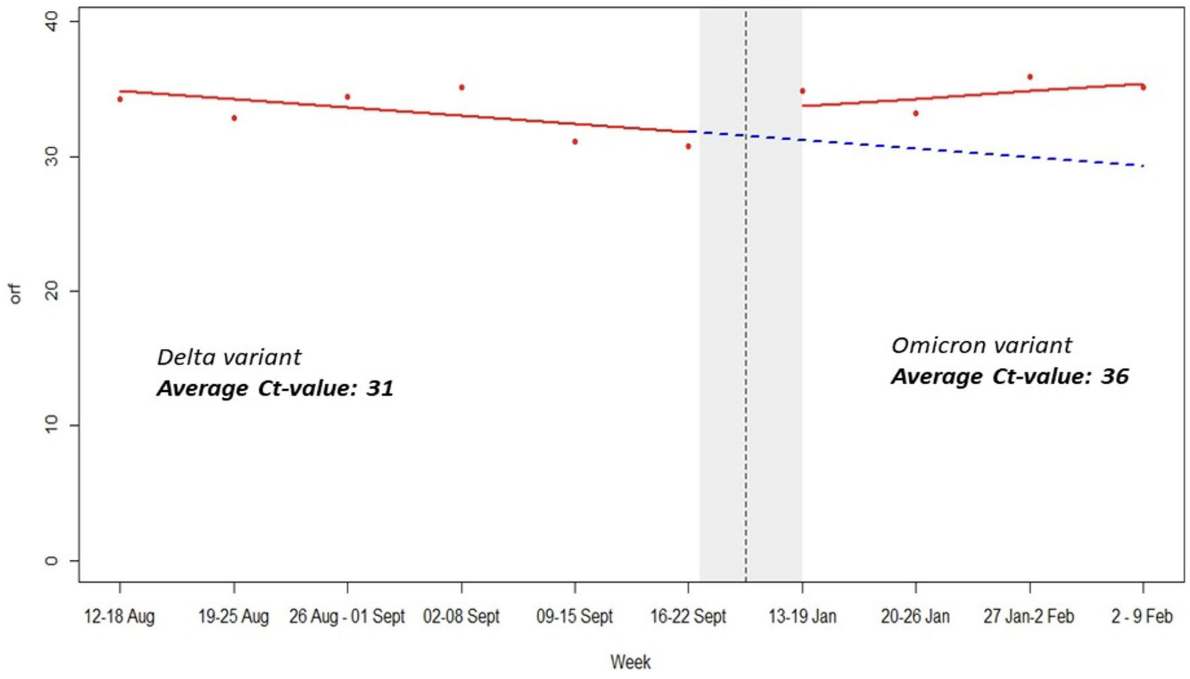

FIGURE 4
Impact of variants C*t*-values on dogs' performance.

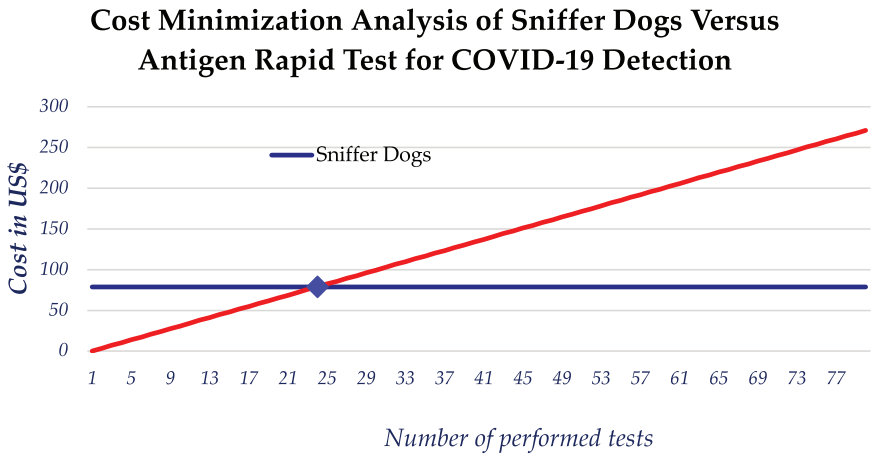

FIGURE 5
Cost minimization analysis between use of scent dogs and rapid antigen tests for detection of COVID-19.

to detect specific odors from individuals with certain types of diseases (7, 10, 11). Other studies examining the capacity of scent detection dogs to detect COVID-19 have reported results ranging from 76 to 100% success rates after 1 week of training (2, 9). Furthermore, the use of scent detection dogs represents a faster, cheaper way of disease detection that requires less technology, minimal training of operators and avoids direct contact between clients and sample collectors, thereby potentially limiting disease spread (12, 13).

In our study of 5,253 samples, detection dogs were able to distinguish infected COVID-19 patients' using armpit sweat samples with good sensitivity and excellent specificity. Our findings indicate that scent detection dogs may contribute effectively to the safe resumption of activities while also helping to keep COVID-19 infections under control. This is especially noteworthy in low-resource settings where testing resources and capacity may be limited. The variation of scent dogs' sensitivity and specificity observed during the two study periods

TABLE 4 Turnaround time estimation for both scent detection dog and RDT per one sample (unit time = minute).

Process	Time for Ag-RDT	Time for scent dog
Preparation of materials for testing	2.00	2.00
Sample coding and registration	2.00	2.00
Sample collection	0.17	0.20
Sample transportation	0.00	1.00
Sample processing and results reading	7.33	0.17
Result recording into the information system	1.00	1.00
Total time	12.50	6.37

is likely explained by the impact of the Delta and Omicron variants. Based on epidemiological data and genomic dynamics of SARS-CoV-2 in Rwanda, the peak of the Delta wave was observed in August 2021 while the peak of the Omicron wave occurred in January 2022 (13). In addition, other factors such as immunity status post-natural infection or vaccination as well as time of diagnostics and sample collection may explain the C*t*-values' variations during both waves. Indeed, during the Delta variant wave, the orf1ab and N-genes' C*t*-values were low while the scent dogs' sensitivity and specificity were 98 and 82.1%, respectively. The period of delta variant wave was characterized by low orf1ab and N genes C*t*-values, severe symptoms, many deaths and high viral load, while the omicron variant wave was marked by high C*t*-values, mild symptoms, low viral load and very few deaths (**Figure 4**). It is important to mention that during the Delta period, the majority of patients who tested positive were symptomatic, likely manifesting in a higher viral load compared to patients who tested positive during the Omicron wave and were often asymptomatic, potentially impacting the detection ability of the dogs. This evidence has been demonstrated by previous studies that have indicated that low C*t*-values are inversely proportional to viral load in COVID-19 patients (9).

Our study findings also indicate that the use of scent detection dogs is cost-effective. Furthermore, scent dogs require limited resources to deploy, and significantly reduce the turnaround time needed to provide results to patients compared to Polymerase Chain Reaction and Antigen Rapid Tests. The cost of mass testing for COVID-19 using scent detection dogs is relatively constant regardless the number of screened persons. Using these dogs during mass testing for COVID-19 would be very beneficial by limiting the cost and responding to the challenge of procurement and distribution of rapid antigen test.

There are some strengths and limitations to this study. The overall strengths include our large sample size obtained from a diverse population across the country, and our inclusion of both symptomatic and asymptomatic patients. Our study also represents the first of its kind to be conducted in a low-income setting, and demonstrates the feasibility of this approach across socio-economic contexts. A limitation of our study is the relatively small number of SARS-CoV-2 positive samples included in our sample due to the successful containment of COVID-19 in Rwanda at the time of our data collection and analysis.

Conclusion

In conclusion, it should be noted that although dogs hold potential as real-time detectors of VOCs, they require intense training and meticulous selection of the best performing dogs before deployment. Interestingly, the variation in dogs' performance could be affected by emerging COVID-19 variants and thus regular refresher training courses are highly recommended for better infection control. Furthermore, as the use of scent detection dogs expands, it is important to take precautions to avoid any risk of contagion while dogs interact with infected human samples. In our study, we designed custom samples holders with double protection systems to protect the dogs from being infected. As the world prepares for future pandemics, trained dogs may offer an important addition to existing diagnostic tools. Subsequent studies could assess the capability of the trained dogs to detect asymptomatic SARS-CoV-2 infection, and then the deployment of dogs in the field and at entry points to support ongoing efforts and COVID-19 response strategies.

Data availability statement

The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

Ethics statement

This study partially involving human participants was reviewed and approved by the Rwanda National Ethics Committee; RNEC/2020-No. 856/RNEC/2021 (FWA assurance no. 00001973-IRB00001497 of IORG0001100). For the use of animals, we did not need an ethics as these were service dogs and this study was approved in accordance with local guidelines. In addition, this study was implemented in the framework of emergency National response to COVID-19 under the coordination of national joint COVID-19 response committee that oversees all COVID response interventions thus did not need a formal ethics approval for use of these service dogs.

Author contributions

LM and SN: conceptualization, funding acquisition, and supervision LM, GM, ErR, HE, ES, PT, RS, CI, JU, DiM, EK, MT,

YB, CM, EdR, FT, SM, AT, RR, MM, VN, BK, CMM, LW, and SN: investigation, methodology, validation, and data curation. LM, GM, ErR, PT, RS, CI, JU, VN, BK, and SN: writing—original draft. LM, GM, EdR, PT, RS, CI, JU, NB, HV, VN, CM, BK, LW, EM, CMM, and SN: writing—review and editing. LM, GM, BK, and SN: project administration. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

Authors LM, YB, and CM were employed by Center for Human Genetics, Inc.

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Expert considerations and consensus for using dogs to detect human SARS-CoV-2-infections

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canine detection, SARS-CoV-2, COVID-19, test system, pandemic control

Introduction

The respiratory coronavirus disease 2019 (COVID-19) caused by the severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) quickly developed into a pandemic (1). Even though laboratory diagnostic tests and vaccines were consequently developed (2, 3), the exploration of rapidly deployable, more reliable tools for addressing the current and future pandemics was vital. Toward this goal, researchers worldwide evaluated the use of medical detection dogs as a rapid, reliable and cost-effective screening method for SARS-CoV-2 infections (4). The ability of dogs to distinguish diseases by their high-resolution sense of smell is based on the volatile organic compound (VOC)-hypothesis (5). Numerous infectious and non-infectious diseases change metabolic processes releasing characteristic VOC-patterns in the form of an "olfactory fingerprint" (6–10). Many studies have shown that dogs can detect metabolic disorders, such as cancer (11) and hypoglycemia (12), predict epileptic seizures (13, 14), or even distinguish various pathogens (8, 15–17). Approximately 78% of the 27 SARS-CoV-2-canine detection studies reviewed by Meller et al. yielded > 80% sensitivity and approximately 60% of studies yielded > 95% of specificity (4), highlighting the potential of the dog as a "diagnostic system" and its recommendation for certain settings. Despite these promising results, all studies published up to now differed in numerous design features. They were mostly designed as pilot studies and case-control selection of patients was mostly favored over a more preferable cross-sectional ("cohort") selection [study quality assessment was conducted and presented by Meller et al. (4)]. The aim of this comprehensive review summary is to provide a general overview of the divergent aspects that may impact canine disease detection and to provide recommendations for future deployment of medical detection dogs (see also summary in **Table 1**). Specific emphasis is placed on the choice of dogs, training paradigms, safety aspects, sample characteristics, pre-screen processing (e.g., inactivation), and screening-population and its environment related aspects, respectively (see also **Figure 1** and **Supplementary Figure 1**), providing an outlook and proposals for the future standardization in the use of dogs for disease detection. Ultimately, this report provides a blueprint for the potential use of medical detection dogs in future epidemics and pandemics.

Disease- or metabolism-derived volatile organic compounds

Infectious and non-infectious diseases can produce metabolic alterations that may be associated with the release of volatile organic compounds (VOCs) from the body (6–10). In this way, specific volatile biochemical fingerprints may be detected and function as biomarkers for corresponding diseases and their clinical course, provided that appropriate sensory means are available (18, 19). The detective olfactory potential of dogs and other animals has been researched in the medical field concerning various infectious viral, bacterial, and parasitic as well as non-infectious diseases and disorders like epilepsy, diabetes, and cancer (5, 11, 20, 21). Horvath et al. demonstrated that dogs can differentiate between normal and neoplastic tissue as well as non-neoplastic disease processes such as inflammation, necrosis or emergence of metabolic products (22). For example, Ehmann et al. reported that detection dogs were able to differentiate lung cancers from chronic obstructive pulmonary disease (COPD) by sniffing the breath (23). The occurrence of specific disease-associated VOC-profiles using chemical analytical methods and technical sensory devices was shown in ovarian (24) and breast cancer (25) or in various respiratory diseases (26) and other infections (27). By applying quantitative analytical methods in animal or *in vitro* models, interesting questions about the temporal and quantitative dynamics of VOC-production across infection states and progress can be addressed. Traxler et al. (28) detected VOC-changes in the breath of pigs after influenza A infection versus control animals. Interestingly, none of the animals in the study displayed clinical signs, indicating that changes in VOCs still remain despite a lack of significant host immune responses (28). Another study measured VOCs produced by B lymphoblastoid cells following infection with specific avian and human influenza strains *in vitro*. VOCs did change depending on infection status, which coincided with the many cellular processes that occur when an organism becomes infected (29).

Gould et al. summarized that, in various viral infections, glycolysis in host cells is elevated due to the necessary energy supply for replication, accompanied with increased production of fatty acids, alkanes and related products (30). SARS-CoV-2-infections were shown to lead to characteristic immune and metabolic dysregulation in proteins and lipids in blood serum (31). SARS-CoV-2-specific biochemical processes, such

TABLE 1 Summarizing comments and recommendations for medical canine scent detection of samples from SARS-CoV-2-infected individuals.

Disease- or metabolism-derived VOCs	Canine detection of SARS-CoV-2-infection is thought to be mainly based on the detection of volatile organic compounds (VOCs). Canine detection of VOCs can occur in real-time with high level of accuracy. However, VOC-detection is susceptible to environmental factors, which can be difficult to standardize. The success of medical detection dogs' VOC-detection depends largely on training with the right variety of target odors.
Ethical considerations	Dogs have different personalities and experiences. They are sentient beings. The learning method should only include positive reinforcement. Dogs can fatigue and get frustrated, which should be considered in the training procedure and when they are deployed in the field. Thus, dogs require adequate work/break cycles and regular positive rewards for their work.
Dog selection	Not only anatomical but also the dog's behavior and personality significantly impacts suitability as a detection dog. Physical and mental fitness as well as high levels of motivation are of crucial importance. Dogs should have a solid willingness to work with humans. Prior detection experience can be helpful.
Dog training	Appropriate training is the key for success in detection. Defining the correct target scent in advance is challenging, especially when the VOC-profile of interest remains unknown. The right grade of olfactory generalization vs. discrimination has to be achieved during training. Sufficient variety of new samples of symptomatic and asymptomatic patients at different stages of the disease process are here required. Duration of training can be variable and should be tailored to the individual dog's success rate. Few days of "retraining" dogs after a longer break are sufficient to reach initial levels of detection accuracy. Line-up, scent-wheel, and detection dog training system (DDTS) have been used for training successfully. Apart from imprinting the specific scent to be recognized, also the search context needs to be trained for. While automated approaches such as DDTS might offer a more randomized and rapid training by providing higher repetition rates, line-up settings are closer to the search context in the field. Blank trials are important in order to test for forced choice decisions and to understand the individual dog's frustration threshold. Dogs should not only be trained with negative samples, but also ideally with samples from other viral respiratory infections to reduce false-positive rates. Further work is needed to standardize and certify training procedures.
Susceptibility of dogs for SARS-CoV-2	Dogs can be infected with SARS-CoV-2, but have a low susceptibility to the virus. Clinical signs are, if at all present, mild. However, biosecurity measures for safe sample presentation, such as virus inactivation and/or safety sample containers during training and/or deployment are recommended, not only for the dogs but also for the handlers.
Sample types	Saliva, sweat, urine, and breath but also respiratory secretions and immediate body odor of SARS-CoV-2-infected individuals express specific COVID-19-associated VOC-profiles, which can be used for training and testing. Sweat collected with cotton pads is not thought to be infectious. Other sample types can be infectious and should be inactivated or presented in a container ensuring biosecurity. Only inactivation procedures should be used, which have shown not to alter the target scent and could bias canine scent detection (see below). Most studies have used sweat samples for practicality reasons. However, it is not clear if cotton-bound VOCs have a similar storage resilience than fluid-bound VOCs such as saliva or urine, which may impact training. Further work is required to provide standardized sample materials.
Virus inactivation	Beta-propiolactone (BPL), heat, ultraviolet radiation (UV), and detergent/solvent are possible measures for virus inactivation. While BPL does not appear to alter canine VOC-detection, heat and detergents might have a greater impact on altering VOC-profiles, which remains ambiguous for UV. However, the use of BPL-inactivation is more time-consuming, requiring laboratories with high safety standards. The least VOC-altering method is to omit inactivation, which works especially well for sweat samples, providing a neglectable risk for infection. In general, biosecurity aspects should never be disregarded and be approved by authorities.
Training sample alternatives	Currently, well-established sample alternatives for a more standardized training for COVID-19-detection do not exist. Artificial "VOC-cocktails," samples from animal models, cell cultures, or pure virus protein are currently being tested and the tests are not yet conclusive. It is likely that proteins can only be used in parts of the training and that the certification procedure will require samples from SARS-CoV-2-infected individuals.
Target population and operational applicability	Studies showed high accuracies for canine COVID-19-detection within seconds with similar or better detection performances than with antigen tests. Depending on disease prevalence and characteristics of the population to be screened, the performance can alter. To ensure certainty in defining the infection/disease status of an individual, multiple back-up dogs can be involved. Changing or distracting environmental factors in the operational setting should be reduced or avoided.

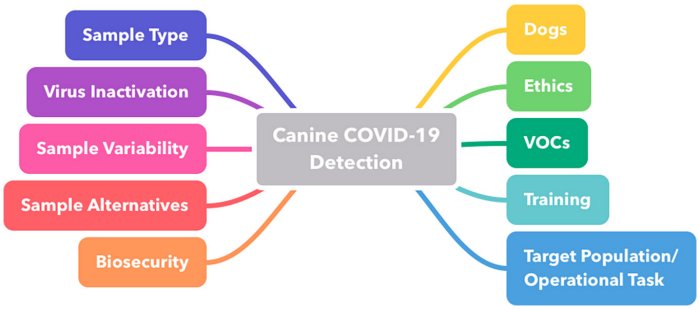

FIGURE 1
Mind map representing key areas of interest highlighted and discussed by the group of experts. VOC, volatile organic compound.

as those associated with modes of entry and replication in cells, combined with induction of humoral and cellular immunologic reactions as well as the dynamic cytokine release might play an important role in COVID-19-specific VOC-expression (32).

The smell of COVID-19

Various studies exist, which give striking insights into SARS-CoV-2-VOC-profiles with differing identifiable VOCs mainly *via* gas chromatography-mass spectrometry (GC-MS), gas chromatography-ion mobility spectrometry (GC-IMS), time-of-flight-mass spectrometry (TOF-MS) or related techniques (33–38). In principle, spectrometric techniques enable the identification and quantification of VOCs in breath samples, preceded by gas chromatographic separation if needed. Prior studies have reported quantifiable differences in about two dozen VOCs between individuals with COVID-19 versus healthy individuals as well as individuals with other respiratory diseases. Particularly striking here are COVID-19-associated elevated concentrations of certain alcohols such as butanol and propanol or derivatives (33, 35, 37, 38), aldehydes such as heptanal, octanal, and nonanal (33, 34, 36), as well as ketones such as acetone and butanone or derivatives (33, 38). Other substances with reported increased concentrations are various alkanes, alkenes, further aldehydes, aromatic substances, and their derivatives (33, 34, 36–38). Decreased VOC-concentrations in COVID-19-breath were shown for methanol (33) and – in contrast to Ruszkiewicz et al. (33) – acetone (35). In addition, Feuerherd et al. showed by headspace air sampling of virus-infected cell cultures that specific differences in 2-butanone, nonane, and pentanal concentrations represent robust discriminatory features between SARS-CoV- 2-, human coronavirus NL63-, and influenza A virus subtype H1N1-infections (39). Similarly, Steppert et al. were able to discriminate between individuals infected with influenza A virus or SARS-CoV-2 analyzing breath samples *via* IMS coupled with a multicapillary column (40). In a study from ten Hagen et al. dogs were able to discriminate supernatants of SARS-CoV-2-infected human cell cultures from 15 other viruses including coronaviridae, orthomyxoviridae, paramyxoviridae, pneumoviridae, adenovirus, and rhinovirus among others (41).

The use of electronic noses (eNoses) has also been explored by some studies for the detection of COVID-19. Sensors and nanotechnology allow to detect differences in the chemical composition of air samples by means of chemical reactions with sensor arrays consisting of specific coatings of certain metal oxides, organic polymers, nanoparticles, etc. (42, 43). The emerging differences in resistance and conductivity produce corresponding "volatile finger-" or "breathprints" *via* artificial neural networks (44). eNoses were able to discriminate breath samples between individuals with symptomatic COVID-19 versus healthy individuals (45–47) or other respiratory diseases (48), Post-COVID-19 condition (49), and non-symptomatic COVID-19 (47, 50). Two recent studies provided evidence that also dogs can detect Post-COVID-19 conditions (51, 52).

Detection of disease-related volatile organic compounds by devices versus dogs

Despite good discriminatory potential within individual studies, the comparison of the described chemical analytical or sensor methods between studies nevertheless highlights some drawbacks of these techniques, which may create challenges for their use in an open screening process. In the following paragraphs, certain features of the canine and technical methods are critically discussed.

First, it is not ensured that all relevant VOCs are reliably detected *via* MS or sensor methods. Differences in databases and small number of metabolites available as standards complicate interpretations of MS analyses (53). Small ions, molecules or molecular fragments cannot be easily detected and make it difficult to interpret and draw conclusions about originally contained compounds. For example, small hydrocarbon-based molecules occur abundantly in exhaled breath, making their detection complicated due to overlap with molecules of similar spectra (38). In addition, certain measurable VOCs are non-specifically altered across diseases making disease discrimination prone to errors. For example, elevated propanol in breath is associated with infectious and non-infectious respiratory diseases other than COVID-19 (35, 54–57). Analogously, a certain "roughness" of detection is also given with eNoses, since the selective and susceptible coatings of the sensors might lead to physical limitations in qualitative and quantitative resolution (42, 58, 59). These aspects become impactful, especially when considering that VOCs in exhaled breath are numerous and most of the VOC-compositions have wide inter-individual variations (60). Similarly, some uncertainties exist in canine detection, as well, since research in perception and processing of certain olfactory cues in dogs is not yet very advanced. Thus, the definition of the target odor, especially in the medical field, remains one of the main challenges in canine scent detection.

Second, differences in the detection of COVID-19-VOCs across studies with MS-detection might emerge due to the choice of different detection and analytical techniques, different patient recruitment procedures and the environment (33, 37). Snitz et al. and Rodriguez-Aguilar et al., who conducted cross-sectional trials in a real-life scenario with eNoses, showed the significant impact of differing sample acquisition methods and environmental factors on the results (45, 50). Although disease discrimination was possible, certain environment-associated deterioration in eNose performance could not be

excluded (50). Therefore, it is probable that the chemical analytical and sensor detection methods are susceptible to "olfactory noise" for COVID-19-detection. While these devices might feed intrinsic and extrinsic VOCs to the analyzer in an unfiltered, noisy, and "one-dimensional" manner, living biosensors such as dogs may perceive the learned sensations that are evoked due to a certain key composition, or "network," of complex and low-concentration VOCs. Dogs are therefore possibly more capable of searching specifically for the "needle in the haystack" than current technical solutions, provided that training samples are correctly and meticulously defined according to the target condition. However, olfactory noise and other distractors may play an important role for canine detection, as well, especially when using detection dogs in the open environmental space. Further research is needed to increase control of these confounding factors.

Third, chemical analytical instruments are often stationary devices. They are mainly used offline and are coupled with software for evaluative steps. eNoses are mobile and online analysis is possible, but they require further software and deep-learning approaches in order to "learn" and analyze specific VOC-patterns. For example, the sensors must be able to detect the correct compounds and in the correct ratio and at low concentrations. The software then has to interpret the signals correctly, and environmental factors can cause difficulties, as described above. Each specific application needs considerable method development work in advance and is cost-intensive which is a drawback in rapid pandemic dynamics of emerging pathogens. Marder et al. stated that "data processing is a major bottleneck of metabolomics" (38). Furthermore, sensors often have a short life and their sensitivity deteriorates in presence of humidity (42, 48, 50). The analysis time for chemical analytical devices or sensors used for COVID-19-detection in the aforementioned studies (see section "The smell of COVID-19") revealed a range of one to 16 min per sample. Dogs, on the other hand, are mobile and can identify COVID-19-samples within a few seconds, i.e., in real-time. This requires preceding specific canine training for high discriminating performance of approximately 4 weeks with a range of 2–15 weeks regardless of the chosen training method (when studies with dogs that had previous COVID-19-scent experience were excluded) (4). However, a variety of factors can have a large influence on learning efficiency, e.g., number of sample exposures, environmental factors, the success of odor generalization, etc. Furthermore, personality traits of dogs and emerging fatigue during work (see also section "Considerations regarding Dog Selection") are impacting factors, which represent a disadvantage compared to well established artificial devices.

Finally, the lower limit of detection in dogs is one part per trillion (ppt), exceeding the range of detection of current available instruments by around three orders of magnitude (61–63). A new study shows that dogs are indeed able to detect even far lower concentrations, in the order of 10^{-21} (Turunen et al., unpublished). Since it was reported that VOCs from breath are released in the range of parts per billion (ppb) to ppt, dogs might appear more suitable for VOC-detection in comparison to instruments with sensitivities in the ppb range (50, 64). However, the canine range of detection was validated in controlled environments, which could mask an actual lower sensitivity. In addition, sensitivity might also depend on the qualitative characteristics of the target odor.

In hospitals and other health care facilities, chemical analytical and sensory instruments are well suited for sensitive and relatively rapid isolation of patients (33, 37), provided that they are swiftly fed with sufficient data for rapid adaptive purposes (36, 65). For external mass screening, the use of such technical devices for VOC-detection is complex due to sample processing time, limited selectivity, and increased susceptibility to material damage as well as to external olfactory noise in a poorly controllable environment. Although similar challenges may exist for dogs, their ability to learn and to process information immediately can make them more capable of searching for specific odors in real time, particularly in complex environments. However, the success of canine detection depends significantly on the training methods and the choice of the right training samples, which is one of the main challenges and disadvantages compared to established analytical and sensory methods. Finally, dogs are likely to be complimentary to sensors and analytical methods and more appropriate for certain scenarios.

Ethical considerations for using detection dogs

One important consideration in repurposing dogs' olfactory abilities for the detection of specific odors is that dogs are living beings with different and individual needs, characters, experiences, behaviors, and capabilities (66). In addition, these elements may differ in the same individual over time due to intrinsic and extrinsic factors. These characteristics, which from an ethical point of view must be protected and respected, considerably distinguish dogs from standardized, industrially produced test kits that have been tailored to a specific purpose (67). Ethical considerations in using dogs' abilities for human purposes are therefore paramount. The method of operant conditioning including positive reinforcement of correct searching behavior by reward (food, toy, etc.) and absence of reward for undesired searching behavior, is considered as ethically unobjectionable, and was the method used across the canine SARS-CoV-2-detection literature (4). For the dogs, the method forms a motivation and pleasure driven detection exercise using olfaction as one of the most important sensory and cognitive tools in macrosmatics. On the other hand, one is confronted with potential short-term issues such as fatigue and/or boredom after a certain time of action, highlighting the

importance of specific and individual adaptations in training and deployment according to the different personality traits of the dogs (67). However, Guest et al. drawing on their experience of deploying medical scent detection dogs, suggest that two trained dogs would have the potential to screen 300 individuals in 30 min in a COVID-19-screening scenario (47), which exceeds the capacity of current available testing methods by far. After the fast screening of a population by canine detection, reference standard reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) of positive scent detected individuals can be applied as further downstream verification (47). Such approaches were already being pursued early in the pandemic, e.g., at Dubai Airport in July 2020.

Importantly, when dogs are deployed in the field and the disease of interest has a low prevalence, reducing the opportunity for the animals to succeed in detection, positive affective and motivational states of dogs have to be sustained in order to avoid frustration (68). This can be achieved, for example, through regular rewards for the respective detection procedure or for detecting specifically prepared (positive) samples (69–71). Interestingly, variation in reward types may lead to a more pronounced maintenance of motivation in some dogs (72). In addition, adequate work/rest cycles for the dogs are of significant relevance for animal welfare and for high efficiency in scent detection work (73, 74).

Considerations regarding dog selection

Besides a well-functioning and harmonic partnership between dog and its handler, many other individual factors can ultimately influence the effectiveness of olfactory detection. These are highlighted in the following. Three recent reviews provide a more detailed overview about the anatomy, physiology, and other factors related to canine olfaction performance (75–77).

Intrinsic factors

Breed-specific anatomy and physiology

The paucity of comparative studies on the olfactory abilities of different dog breeds including intra-breed variations represents a challenge for the selection of suitable detection dogs (75, 76). Although it can be hypothesized that anatomical and physiological characteristics of the olfactory organ play a crucial role, behavioristic and mental aspects, personal traits, and experiences are of no less importance for adequate canine screening work (76).

The mechanisms involved in molecular recognition in olfactory receptors (OR) and olfactory sensory neurons and in the identification of specific odorants are still only partially understood. In this regard, the current consensus is that each OR has a characteristic ligand spectrum and each odorant can also be detected by a combination of ORs (78). Gene polymorphisms in expression of ORs in the same breed but also between breeds differ and may be used as an indicator for scent discrimination performance (79–83). In addition, the total number of neurons, i.e., the size of the olfactory epithelium, may have an effect on olfactory acuity in dogs (79), which might be due to enhanced olfactory resolution with increased numbers of neurons (84). One study showed that dolichocephalic or normocephalic dogs (often classified as scent breeds) and wolves have better olfactory capabilities than non-scent breeds and brachycephalic dogs (85). Brachycephalic breeds have less space for the olfactory epithelium to expand in the nasal cavity and less olfactory cells reducing olfactory sensitivity, and pronounced breathing issues leading to reduced cerebral oxygen supply, reduced heat elimination, and therefore to quicker fatigue (84, 86, 87). Thickened conchae and less ramifications inside the nasal cavity may be a reason for less epithelial surface (88). Brachycephalic breeds should therefore be avoided for scent detection tasks (76). Controversially and surprisingly, Hall et al. showed that pugs are able to outperform German shepherds in olfactory tasks (89), highlighting that behavioral aspects play a crucial role as well.

Furthermore, olfactory airflow in dogs in ethmoidal regions is laminar which is optimized for scent molecule transport (62, 90). This type of airflow is impacted in brachycephalic breeds due to an obstructive and deforming development of the nasal cavity and nasal conchae (87, 88), especially since the dorsal meatus in the canine nasal cavity, functioning as a bypass for olfactory laminar air supply, is only ventilated when high inspiratory pressure is applied (62, 90, 91). However, Wagner and Ruf showed that a large surface of the bony turbinates in dolichocephalic dogs is not the main reason for a better smelling ability (92), highlighting, again, the fact that breed and anatomy should not be the ultimate reason for defining a good detection dog (76).

All mentioned dog breeds in the reviewed literature of canine COVID-19-detection by Meller et al. were normocephalic breeds, which are typically used for scent detection work and are known for their outstanding olfactory capabilities and resilience (e.g., Belgian Malinois, German Shepherd, Labrador Retriever) (4).

Dog health, behavior, and sex

Impact of physical, behavioral, and sex related factors are less investigated than anatomical properties. In addition to a well-functioning olfactory system, a high degree of physical and mental fitness and especially motivation are essential for dogs to focus on the target scent in different environments (76, 93). Although the speed of dogs' olfactory system is currently unsurpassable by itself, a high level of stamina, agility, athleticism, and motivation in dogs is of great benefit to enhance

testing throughput. A high motivation and fitness level can compensate for difficulties in search tasks that prove fatiguing and where target odors are scarce (94–96).

Good cooperative work with humans, especially a balance between obedience and independence, is essential in deployed dogs. On the one hand, independence ensures self-determined searching strategies rather than being potentially misguided by the dog handler. On the other hand, obedience leads to efficiency, where the dog handler can narrow down the area for the search (76).

Aggression toward humans and other animals should be excluded and distractibility and anxiety levels should be as low as possible. Female dogs are generally considered less aggressive and more cooperative (97–99). In terms of neurophysiology, cells in the olfactory bulb of female dogs were shown to be more active than in males. Furthermore, female dogs have a better long-term memory (100). Neutering was assumed to decrease levels of aggressiveness and distractibility (101, 102) and males were shown to perform better in terms of directionality assessment of the target odor (103). Importantly, however, Jamieson et al. summarized that breed and sex finally should not be the crucial cornerstones to assess suitability of detection dogs since training, socialization, experience, and long-term and short-term environmental exposure can have far-reaching influences (76). Sex aspects and potential differences in terms of olfactory performance were not relevant in the screened COVID-19-detection studies (4).

Physical health of deployed dogs should always be guaranteed in the first place from the perspective of ethics and animal welfare and, secondly, not to interfere with their scent performance. Diseases and disorders capable of impacting canine olfaction are, e.g., tumors and injuries in the nasal cavity, infections like aspergillosis, distemper and parainfluenza as well as endocrinological disorders like hyperadrenocorticism, hypothyroidism, and diabetes (104, 105). Also, the function of the vomeronasal organ, supposed to be responsible for detection of pheromones and low-volatile substances, can be impacted by diseases (77). A parotitis was shown to decrease the accuracy of SARS-CoV-2-detection in one study dog (69).

SARS-CoV-2-infections can affect olfaction in people (106) and in some animal models [e.g., hamster (107) and mouse (108)]. Although dogs can be infected by SARS-CoV-2 (109, 110), typically, no clinical signs or mild and reversible signs are observed and susceptibility appears to be low (111). However, biosafety measures should be used when deploying scent detection dog teams (see also sections "Susceptibility of dogs for SARS-CoV-2" and "No viral inactivation").

Dog mental condition and age

Olfaction is affected by aging processes, and can manifest in atrophic degeneration of the olfactory epithelium, decreased neurogenesis and loss of olfactory cells and their cilia (112). Wells and Hepper showed that younger dogs perform better in olfactory directionality perception than older dogs (103). In addition, pathological aging (e.g., canine cognitive dysfunction) is associated with lower olfactory capabilities (113–115), analogous to human patients with Alzheimer's disease (116). In 27 studies reviewed by Meller et al. (4) the median age of dogs involved in COVID-19-detection was 3 years (range 0.5–12.0). Age dependent canine olfactory performance in COVID-19-detection cannot be provided as such comparisons were not in the scope of the reviewed studies (4).

Extrinsic factors

Prior scent detection experience

Previous detection experience in dogs is of great advantage. However, inexperienced dogs can be trained and deployed for COVID-19-detection, as well, achieving high diagnostic accuracies (4). For example, Chaber et al. showed that inexperienced dogs were as efficient and accurate as experienced dogs (117). Experienced dogs that are accustomed to the mechanics and environment of odor detection tasks, may only need to learn the new target odor-profile, whereas more time must be allowed for inexperienced dogs to learn to handle the procedure in the setting confidently and efficiently. However, more time should be calculated when changes in setting between training and testing occur (71, 118), even for experienced dogs, in order to ensure understanding of the new search context. Interestingly, the experience of dogs in detecting odors seems to be positively correlated with the ability to cope with more complicated odor information and with stability of long-term memory (100). Inexperienced dogs seem to use olfaction to a lesser extent than experienced dogs (119), highlighting that olfaction is subject to learning processes and plasticity and can be shaped accordingly. Therefore, frequent olfactory exercises with alternating scenarios are beneficial to the training repertoire and experience.

Dog operational environment

High environmental humidity seems to be favorable for scent perception in dogs probably due to increased nasal humidity and enhanced odorant trapping (120), while high temperatures might have a negative impact on the general work flow (121). The dehydration of the mucosal layer in the dog's nose can decrease the odor detection capabilities (122). The training and sample assessment by the dogs is preferably done in a spacious and conditioned room with controlled temperature and humidity. Still, dogs should be let out in between runs to avoid boredom and increase their odor detection capacity. It was found that dogs had a lower performance when they were exposed to direct sunlight and at higher temperatures (Callewaert et al., in preparation). Kokocińska-Kusiak et al. described environmental factors concerning canine scent detection in the open field, highlighting how sudden

environmental changes might impact olfactory abilities (77). However, even in a more spatially restricted searching context, where dogs are involved as screening tools, alterations of external factors should be kept to a minimum. In the study of Vesga et al., COVID-19-detection dogs directly sniffing people in public transport performed at 69% sensitivity. However, those dogs had no training for 2.5 months prior to this testing scenario and still performed well (118). In the study of ten Hagen et al., dogs had high detection accuracy in line-up screenings at concerts (sweat samples, sensitivity 82%, specificity 100%) following the training phase of one to two weeks in a line-up setting (71). Apparently, dogs' performance was not affected by potentially constantly changing odor-profiles at the testing location, although testing was restricted to a dedicated, roofed, and protected area (71). It may be probable, that a setting of stationary and somewhat isolated dog detection procedures in the form of a checkpoint, e.g., in an isolated roofed space, is more suitable, efficient, and more constant than letting dogs pass through crowds of individuals where olfactory and further environmental sensory distractions may have a higher impact (69, 123). Interestingly, there are also specific and rigorous training programs for explosive detection dogs (e.g., Vapor Wake®dogs) designed for the reliable detection of odorants in aerodynamic wakes of moving individuals in crowds of people (124).

Other external influences on canine olfaction performance can originate from food and drugs. Certain food compositions and ingredients can enhance or decrease olfactory acuity (120), which seems to be dependent on the level of physical exercise in dogs. Angle et al. found benefits to olfactory performance when corn oil supplemented diets were used together with exercise (125), whereas feeding coconut oil supplemented diets without exercise impaired olfaction (126). Interestingly, relatively few studies exist concerning commonly used drugs in dogs and their impact on olfactory performance (77). Especially, metronidazole (127) and steroids like dexamethasone or hydrocortisone (128) have the potential to impair olfaction.

Considerations regarding dog training

Training is the most critical step in predicting the success of dogs in any form of detection work. Dogs without prior odor detection training must learn the value of odor detection, associating a reward with the smell of the target sample. The physical mechanics of searching for and responding to odor in the training and testing environment may be novel to dogs, even to those with prior odor detection experience. Many dogs trained in odor detection (e.g., explosives, narcotics) are trained to recognize an odor, but are not required to discriminate between two very similar odors (i.e., human scent from a diseased state versus human scent from a non-diseased state). Therefore, dogs must learn that the background scent (i.e., individual people) can vary greatly, but the target is the common scent present in only diseased individuals, a task which requires generalization (129). Thus, defining the correct target scent in advance is crucial for the training and subsequent testing in the field (see section "Variability of samples"). Because little is yet known about the COVID-19-odor, target scent definition may seem inconsistent, especially early in a pandemic. Nevertheless, the majority of dogs involved in COVID-19-screening studies performed with high diagnostic accuracies with novel samples in the diagnostic test evaluations (DTEs) (4).

The training method used across COVID-19-studies was operant conditioning with positive reinforcement of correct searching and indication behavior using reward (food, toy, etc.) and the classical conditioning for odor imprinting (presentation and conditioning of the target scent). This method allows for an intrinsically arising motivational boost, which is the determining factor for successful learning. However, training protocols differ depending on the materials, settings, and learning approaches that were used (4). Therefore, there is a lack of standardization of canine training methods for disease recognition, especially for COVID-19, resulting in uncertainty in intra- and inter-dog reproducibility and in translation to real-world scenarios (130). Currently, standardization methods are being developed and a detailed training protocol is provided as supplemental material by Chaber et al. (117). Furthermore, ten Hagen et al. emphasized that integrating other, similarly acting pathogens into training procedures is reasonable in order to decrease the false positive rate and to sharpen the accuracy of dogs for SARS-CoV-2-detection (41). Once dogs learn to reject samples of similar pathogens that appear frequently in a population, sharper discrimination between these pathogens and the target pathogen can be achieved (41).

Olfactory generalization

A key component for consideration during the training process is the scent generalization, which ensures that the dog searches for the common scent-profile of a target condition among all samples of interest rather than recognizing individuals (129). The degree to which generalization is required also depends on the search context. When deploying dogs as a pandemic countermeasure, exposing the dogs to numerous and varied samples from both affected and unaffected individuals will likely lead to higher proportions of correct decisions in an open field screening-scenario, where sources of olfactory confounding factors may be numerous (e.g., age, physiological condition, other diseases, diets, hygiene, habits, environment, etc.). However, too broad of a generalization gradient can also lead to a "dilution effect" of the target scent perception. In this condition, a wide range of different odors is present in the learning repertoire, which differ gradually from the target odor. Thus, too much generalization may mean that dogs also recognize odor-profiles that are merely COVID-19- or

SARS-CoV-2-associated. This would lead to an increased false-positive screening rate. Training on a narrow and invariable scent repertoire, on the other hand, can lead to increased discrimination and to confident recognition of very explicit odor patterns or individual samples. This situation can be a problem for screening of a disease-associated odor-profile among plethora of individual odors, potentially leading to an increased false-negative screening rate (129). The main challenge in canine medical scent detection is to assess the origin of the olfactory profile of interest through a myriad of metabolic and other processes, and thereby to define the target odor. The lack of knowledge about the exact odor-profile of COVID-19 and whether this odor-profile is consistent among individuals, represents a "black box" for dog training and makes balanced generalization very challenging. Both balanced generalization and discrimination can be useful, depending on the search context, to enable multi-layered searches, e.g., starting with a broad screening by dog x (e.g., condition) followed by a specific search for a particular target (e.g., pathogen or variant) by dog y (see also section "Standardized sample alternatives"). In order to assess adequate degrees of generalization dogs should be regularly confronted with new samples, both during training and, more importantly, when DTE is conducted. Dogs' reaction should always be carefully observed, especially when confronted with novel samples, to determine whether generalization processes took place. However, the exact mechanisms of olfactory generalization remain poorly understood (129). An interesting contribution could be made by studies that titrate the intensity of generalization upon detection of disease odor against dogs' performance. In this way, rates of correct choices for defined samples, or a defined condition, could be compared between dogs trained with different odor-profiles varying in their odorant spectrum. This could provide important conclusions about the relative generalization process. However, it is probable that generalization depends on further properties of odorants, e.g., source, quality, and quantity of odorants, interactions between odorants, etc., as well as on the individual dog's personality or learning style.

Training duration

Training periods varied between canine COVID-19-detection studies as durations were chosen arbitrarily. Overall, dogs were trained in 2–15 weeks (median 4 weeks), including habituation (e.g., familiarization with scent work, search contexts, and workflow) and/or imprinting, for the detection of COVID-19- or SARS-CoV-2-infections, if no prior COVID-19-scent experience was present. No systematic testing for detection accuracy after different previously defined training periods has been reported and typically the increasing training performance over time was used as a basis for the decision to start the DTE (4). Vesga et al. showed that dogs still performed in an acceptable way (69% sensitivity, 94% specificity) after a training gap of 2.5 months (118). Interestingly, half a week of robust "retraining" of dogs with previous COVID-19-detection experience resulted in comparable high COVID-19-detecting performance as observed after initial training (41, 52, 71). In contrast to the results in the study of Vesga et al. (118), a recently published study highlighted that dogs indeed can remember at least 40 different defined odors, not experienced within 12 months, with 100% accuracy (131). However, the metabolism-induced smell of a disease may be more complex and more difficult to detect (and to remember) than more simple odors, especially among numerous individuals. In addition, it appears important to regularly confront dogs with fresh samples in order to react dynamically to changing disease conditions (e.g., new virus variants) early and reliably. Further research is necessary to assess the potential of canine olfactory memory in order to establish efficient training and break plans for the maintenance of high olfactory performance and for the reduction of fatigue- and boredom-related performance losses.

Training setting

The majority of the 27 reviewed studies by Meller et al. (4) used training with line-ups (19 studies; **Figure 2**). Scent-wheel training (**Figure 3**) was used in three studies while five studies used the Detection Dog Training System (DDTS; **Figure 4**), a device dedicated to the automated, randomized and software-driven presentation of samples (4). In the more classical training methods, great care needs to be taken when exchanging the scent containers, so that sequencing is randomized and blinding of the study is guaranteed which, in addition, requires sufficient personnel and material. Manual and frequent exchange of containers is time-consuming and contamination of containers needs to be avoided. In case of reusable sample containers cleaning after usage and between sessions with different dogs is of crucial importance. While in the traditional approach the dog usually works together with its handler, in the DDTS-approach the dog works independently, significantly decreasing handler bias ["Clever-Hans"-effect (132)], and the sample presentation frequency is high with multiple presentations per minute and an automated reward system. The studies that used DDTS observed generalization quickly despite a limited number of samples used (41, 52, 71, 133, 134). While such automated approaches might enable fast scent conditioning (135), they lack the reference to real-life scenarios where samples (or individuals) would be presented along a line (e.g., airports, schools, events, etc.) to the dog and its handler. Therefore, it is recommended to use a mixed approach using automated methods for initial fast, unbiased scent imprinting and generalization with subsequent habituation and training at line-up or scent-wheel settings to train dogs for systematic and controlled screening in real-life scenarios. It is also recommended to regularly challenge dogs in training with "blank trials". These trials, which do not contain samples with the target odor, are conducted to evaluate whether

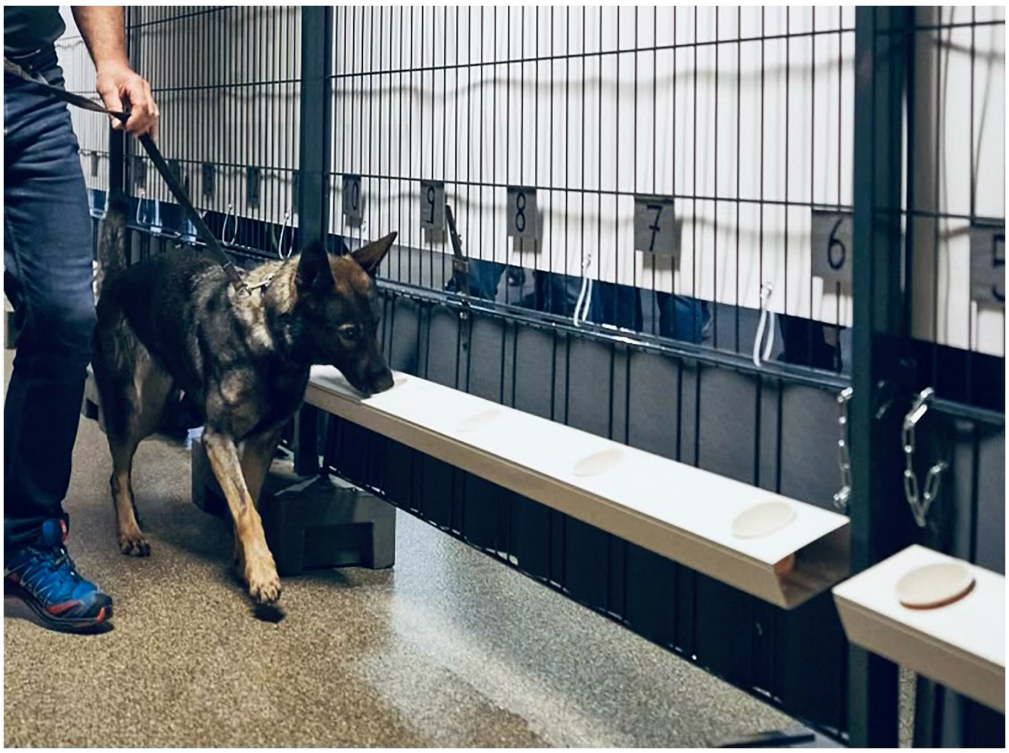

FIGURE 2
Dog and its handler working in a line-up setting.

dogs perform forced choice decisions, possibly due to rapid frustration after not finding the target odor. Especially, when prevalence of a certain disease is low, such frustration thresholds in detection dogs must be high and monitored. Based on the frustration level of the individual dog, target scent samples should be presented at a dog specific interval to keep frustration levels low (see also section "Ethical Considerations for Using Detection Dogs"). Of the 27 reviewed studies by Meller et al., only nine studies reported the use of blank trials (4).

Susceptibility of dogs for SARS-CoV-2

To date, little is known about the susceptibility of dogs to SARS-CoV-2 and the disease caused by it but initial findings indicate that the susceptibility is low (111). However, studies have shown that dogs can be infected, accompanied by seroconversion, but usually do not show symptoms of disease (110, 136–140). However, a rare association between SARS-CoV-2-infections and the development of myocarditis has been suggested (141). Virus shedding seems to occur to a small extent due to limited titers and during a very short period of time (136, 139, 140). In addition, infection appears to be complicated because only a small percentage of dogs living in households of COVID-19 patients become infected (110, 137, 138). Therefore, there is currently no evidence that dogs play a determining role in virus circulation or transmission to humans, but this should not be ruled out at this stage (110). In contrast to dogs, ferrets and cats seem to be more susceptible to SARS-CoV-2-infections (111, 142, 143).

In the reviewed studies of canine COVID-19-detection, there are no reports of SARS-CoV-2-infections in the involved dogs (4). Of the three studies that conducted PCR-testing of the dogs after the tasks, none of the dogs tested positive (118, 134, 144). However, those studies had high biosafety standards. Biosafety measures should be addressed in training and testing, such as safety containers (118, 134, 145) or chemical and physical viral inactivation measures (41, 52, 71, 133, 134, 145–147). In addition, personal protective equipment should be used to protect involved individuals, even in the case that samples show low infectivity (see section "Sample types"). The following sections discuss the wide variability of sample types and inactivation measures used in reviewed canine COVID-19-detection reports (4).

Samples for use in training and testing

Upper respiratory tract samples like nasopharyngeal (NPS) or oropharyngeal (OPS) swabs and, under certain

FIGURE 3
Dog working at a scent-wheel.

circumstances, lower respiratory tract samples (e.g., tracheobronchial aspirates) are routinely used for the detection of viral nucleic acid *via* PCR-techniques (148). Especially RT-qPCR as well as lateral flow immunoassays (LFIA) are currently widely used to identify ongoing infections and rely on the direct detection of viral presence by identifying certain nucleic acids or antigens, respectively (149). Temporal and quantitative presence of SARS-CoV-2-RNA detected *via* RT-qPCR varies across different human biological sample types and across the duration of infection (150, 151).

Although the virus is essential for the induction of VOCs, the metabolic changes detected by dogs are not necessarily linked to the persistence of the virus, neither locally nor temporally, and VOC-release may lag or precede detectable viral infection (51, 52). A global COVID-19-VOC-profile affecting the whole organism seems to be plausible since Jendrny et al. could show that dogs were able to detect SARS-CoV-2-infections in different body fluids although being trained with only one sample type (134). How essential VOCs change over the time course, disease state, and other disease characteristics still needs to be elucidated. Nevertheless, if biological samples are used for training, it is of crucial importance to "capture" the odor-profile related to the operational usage, e.g., acute and active infections, since only then dogs can be involved as screening tools.

The following sections will give a brief overview of sample types used in the reviewed COVID-19-scent dog literature by Meller et al. (4). Sample handling and options for preserving VOCs in samples (e.g., storage, etc.) can be found in the individual study protocols.

Sample types

Saliva and respiratory secretions

Most studies comparing viral content in saliva and respiratory samples showed saliva samples to contain SARS-CoV-2-RNA in patients of differing age and with differing severities of COVID-19-infections (152). Saliva may substantially contribute to the airborne/droplet transmission (153, 154). It is suggested that in the oral cavity and in epithelial cells of minor salivary gland ducts significant expression of angiotensin-converting enzyme 2 (ACE2) and transmembrane protease serine subtype 2 (TMPRSS2) may contribute to enhanced viral invasion of the host cells by coronaviruses

FIGURE 4
Dog working at an automated detection dog training system (DDTS).

(154–157). High levels of SARS-CoV-2 in saliva are usually already detectable at COVID-19-symptom onset, and usually the loads are similar or slightly lower than in NPS/OPS (151, 152, 158–164). Some studies showed higher viral loads in saliva in some patients or positive tested saliva samples while NPS/OPS presented negative (163, 165–167), which could be due to poor NPS/OPS sampling quality or due to earlier viral manifestation in the oral cavity (165). Nevertheless, a significant decline of viral loads in saliva takes place in the later time points of infection compared to NPS/OPS (151, 158, 159, 162, 164). Interestingly, the grade of salivary viral load does not seem to be associated with disease states (158, 168).

For scent dog detection, saliva was used in the DTEs of six of the reviewed studies, whereas upper airway samples were used in four of the 27 studies (4). Saliva is relatively easy and quick to obtain, but can contain high loads of viable SARS-CoV-2 in infected individuals. Therefore, samples have to be inactivated or presented in a high-security setting in order to protect dogs and their handlers from infection (see section "Pre-processing of samples"). These crucial steps can considerably complicate the training. Jendrny et al. showed that dogs trained with beta-propiolactone (BPL)-inactivated saliva samples can transfer their gained olfactory abilities to the detection of previously unknown non-inactivated SARS-CoV-2-positive saliva samples, and even to previously unknown non-inactivated SARS-CoV-2-positive sweat and urine samples (134). This successful transfer performance simplifies the training (and the real scenario deployment) considerably as it can be extrapolated from the results that regardless of the training samples used, COVID-19 can be detected by trained dogs in the real screening scenario based on a global and specific disease odor. Similarly, Essler et al. showed canine transfer abilities between urine and saliva as well (145). These results support the GC-MS-based studies by Penn et al. and Soini et al. who revealed that general VOC-compositions in human saliva and sweat overlap to a large extent (169, 170). The probable direct infection of the epithelial cells in the salivary gland ducts would provide a high grade of COVID-19-associated VOCs dissolved in saliva and further secretions from the oral cavity functioning as stable carrier media. However, it remains to be elucidated if the fluid-bound condition in saliva might elongate VOC-presence in contrast to non-fluid-bound VOCs as it occurs in sweat/body odor samples. Interestingly, research in biomarkers established the term "salivaomics" since composition of saliva appears to be sensitive to differing disease states of the organism (171). Therefore, it can be speculated that the metabolism-based olfactory fingerprint of COVID-19 in saliva has a relatively specific representation. Further important questions to elucidate are under what conditions and how long saliva samples can

be stored without significant loss of characteristic COVID-19-VOCs, and how VOC-production and -dynamics are related to the temporal and clinical course of the infection. One of the authors successfully used frozen aliquots of BPL-inactivated saliva samples for training purposes within a year with success (personal communication).

Sweat and body odor

In contrast to the biological material from the respiratory tract, the potential for SARS-CoV-2-infectivity *via* sweat or skin is considered negligible. However, based on research on previously described human beta-coronaviruses, attention should be drawn to sweat as one possible vehicle of SARS-CoV-2-transmission (172). Skin, sweat and sebaceous glands express ACE2-receptors (173, 174), making SARS-CoV-2-infections of the resident cells probable (175, 176). However, viral load in epidermis and sebaceous glands was shown to be extremely low by immunohistochemical analysis (175, 177, 178). In contrast, cells in sweat glands contained high levels of viral spike proteins whereas cells in the sweat ducts contained low levels (175). Recalcati et al. tested the sweat of 22 hospitalized COVID-19 patients, the sweat of only five patients was SARS-CoV-2-positive *via* RT-PCR (179). In contrast, Arslan et al. did not detect viral nucleic acids in multiple sweat samples from both axilla and forehead in 50 patients with COVID-19 (180). Similarly, Fathizadeh et al. did not detect SARS-CoV-2 in sweat from the forehead of 25 patients with COVID-19 (181). These results indicate that despite potential viral presence in sweat glands, viral shedding through skin and sweat is unlikely (but not impossible), allowing for less strict security measures concerning sweat/body odor samples.

Sweat/body odor on pads, gauze, etc., or clothes was used in the DTEs of 20 of the 27 reviewed studies, while direct sniffing of live humans was conducted in only one study (4). Sweat and skin surface also appear to release VOCs which may vary depending on the internal health state of the organism (18). However, bacteria on the skin surface can also influence the metabolism of the released VOCs (6). Whether the composition of the microbiome on the skin has an impact on the COVID-19-associated VOC-profile, and whether these variations may alter scent dog acuity, needs to be elucidated. Nevertheless, body parts frequently or constantly exposed to personal care products, cosmetics, and perfumes are not ideal for sample acquisition since interactions of these products with bacteria and VOCs can occur. Furthermore, clothes intended for SARS-CoV-2-detection by scent dogs should not be washed before being presented to the dogs since important VOCs like organic acids would be destroyed by this process (182). Importantly, the non-homogeneous distribution of apocrine and eccrine glands in the skin implicates different compositions of VOCs depending on the sampled body region (183). Indeed, a varying collection period was applied depending on the body region across the reviewed COVID-19-detection studies. While a short swabbing of the crook of the arm, wrist, face or neck was sufficient for high diagnostic accuracies with sensitivities and specificities above 91% in three studies (69, 70, 134), studies that used axillary sweat or other sweat type chose a longer collection period of around 1–20 min (51, 117, 184–192) or even periods of hours in case of clothes (47, 144). However, in most cases these periods were arbitrarily chosen (4). Callewaert et al. (in preparation) found that 30 min sampling of the underarm skin yielded better canine results versus 15 min sampling.

Compared to saliva or urine sampling and processing, sampling of sweat/body odor on cotton pads or clothes represents a quicker, safer, and more feasible method without inactivation procedures and is well suited for rapid scent dog mass screening. However, it is unclear whether VOCs have a comparable half-life on solid materials such as cotton pads compared to liquids. This could complicate the creation of a long-lasting training sample set if not stored appropriately, but this remains speculative and needs to be elucidated in future studies. For example, Gokool et al. provided preliminary evidence that the specific odor persists for months in worn cotton shirts (193). Sweat samples for COVID-19-detection were stored cooled or at room temperature for around 2 h (194), 24–72 h (70, 185–187, 189, 190, 192), 1 week (188), or even up to 6 months in triple zip-lock plastic bags (69) before being presented to a dog. In some studies, sweat samples (and clothes) were frozen and then presented to the dogs thawed after longer storage periods in order to preserve VOCs (47, 52, 117, 134, 191). Those qualitative and temporal differences in storage did not seem to impact canine performance (4). A combination of training with inactivated saliva or other liquid-bound respiratory material with a stable VOC-profile and testing with rapidly obtainable sweat samples in a real-life scenario could be an effective, safe, and sustainable learning and testing method for infectious disease testing. However, an additional challenge with fresh sweat samples during training is recommended.

Urine

Viable SARS-CoV-2 or its RNA was detected in urine of infected individuals in various studies (153, 195–197). Although some studies showed no detectable virus in urine (198, 199) or, at least, very low viral loads compared to respiratory samples (200), other studies suggested similar viral loads in both sample types (153). These discrepancies might suggest that urinary transmission of SARS-CoV-2 is less likely in general, but that dynamics of viral shedding *via* urine could be highly dependent on the clinical and temporal stage of the disease (153, 200–203). These concepts are supported by a longitudinal study from Joukar et al. (204) who showed that at clinic admission of COVID-19 patients (n = 100), only 7% of the urinary RT-PCR-tests were positive. The maximal duration of viral persistence in urine was 11 days post admission which was shorter than for all other examined sample types (204). Similarly, Yoon et al. revealed a rapid decline of viral loads in urine to levels below

detection limit after only 3 days post admission (151), narrowing the temporal window of virus detection in urine (202). Possible enhanced viral infections of the urogenital tract are plausible due to a prominent expression of ACE2 and TMPRSS2 (205) and renal abnormalities due to SARS-CoV-2-infections cannot be excluded (206, 207).

Urine was used in the DTEs of three studies (52, 134, 145) of the reviewed COVID-19-scent dog detection studies by Meller et al. (4). Chemical analyses of VOCs in urine have been used to detect olfactory fingerprints of different types of cancer (208) and bacterial infections like tuberculosis (209, 210). Since urine contains the intermediate or end products of numerous converging metabolic pathways it can be considered a VOC-rich body fluid (6), although saliva contains a larger variety of VOCs (208). However, due to glomerular filtration VOCs might be more concentrated in the urine than in other body fluids (211). Interestingly, in a direct comparison between saliva, sweat and urine, dogs were able to detect urine from COVID-19 patients with high certainty (median sensitivity 96% and specificity 98%), although the dogs had been trained with saliva beforehand. This might indicate a high concentration of COVID-19-associated VOCs in urine (134). Urine sampling requires more infrastructure, time, and effort and, therefore, is less suitable for mass screening scenarios compared to saliva or sweat. In addition, viral inactivation or high-security measurements should be considered due to the potential risk of viral transmission. Due to the high detection accuracy achieved in the study from Jendrny et al. (134), testing of urine could be used for additional *post hoc* confirmation after detection of a positive case during screening with other sample types. As with other sample types, however, optimized storage properties still need to be investigated. Furthermore, aspects like diet can impact urinary VOCs significantly (6), which needs to be addressed in future studies.

Breath

Exhaled breath contains high concentrations of various particles and molecules (212–214) including VOCs (60), with differing compositions among certain pathological conditions (6, 215). Although "violent" expiratory events such as coughing and sneezing have previously been considered the main contributors to infectious aerosol and droplet infections (216), aerosols generated by breathing can transmit SARS-CoV-2 and may have a major impact on the infection dynamics (217, 218). Breath VOCs were already investigated in many other diseases (19, 219) and initial approaches have been made in COVID-19 (see also section "The smell of COVID-19").

Breath samples were used in the DTEs of six of the COVID-19-detecting dog studies, especially in combination with masks (4). The collection and conservation of VOCs from breath is challenging. Lomonaco et al. showed that general VOCs of breath samples stored in sorbent tubes at room temperature were stable up to 72 h (220). A study by Kang and Thomas stated that significant loss in some endogenous breath VOCs was already discernible after 6 months of −80°C storage, although specialized adsorbent tubes were used (221). It is therefore probable that VOCs in masks, similar to cotton pads or clothes, have a storage resilience of shorter duration. However, Guest et al. showed that clothes (socks) gave a stronger specific olfactory signature for dogs than breath samples (face masks) (47). This suggests a high inter-individual VOC-variability in breath samples (60) (see also section "Detection of disease-related VOCs by devices versus dogs"). Furthermore, higher storage temperatures drive a greater loss of breath VOCs on adsorbent materials (222) and longer storage times can lead to exogenous contamination (223). These properties impair the establishment of stable breath sample sets for training. On the other side, subtle skin abrasions in masks, cotton pads, and clothes certainly contribute to a prolonged retention of certain VOC-profiles (see also section "Sweat and body odor") (6). Due to the impressive acuity of canine olfaction, the potential storage artifacts of breath samples might represent a negligible drawback, this issue however has to be addressed in further studies.

Unlike eNoses, into which the breath sample is usually fed directly, the direct presentation of pure breath in training and in real-life screening to the dogs is challenging, which is not the case for solid-/adsorbent- or fluid-bound biological material. Furthermore, due to technical and hygienic reasons, the throughput rate of current eNoses in real-life screening is lower (minutes per sample) than the throughput rate of trained dogs evaluating line-ups with self-taken sweat samples (seconds per sample) (69, 71) (see also sections "Detection of disease-related VOCs by devices versus dogs" and "Sweat and body odor"). In terms of breath VOCs, masks (or specialized adsorbent material) would be more suitable than pure exhaled breath both for canine training and screening. However, generating those mask samples generally required a longer duration of approximately 10 min to 24 h with a median of 180 min (47, 144, 146, 147, 190). Vlachová et al. however, conducted breath sampling on sterile surgical compresses of only 3 min (189). Furthermore, due to the evidence of airborne/droplet infections for SARS-CoV-2, biosecurity associated with the immediate presentation of pure breath samples is more complex than presentation of carrier material-bound samples.

Variability of samples

Origin of samples for training and DTE purposes is an important factor due to the potential contaminating impact of environmental VOCs (6, 208, 215, 224). For example, it could be a major issue if samples from SARS-CoV-2-positive patients originated from only one facility, conditioning dogs on facility-associated smell rather than SARS-CoV-2-associated smell. Likewise, if SARS-CoV-2-negative patients are collected from a different environment, e.g., the community, and SARS-CoV-2-positive patients are all collected from hospital environments,

the systematic difference between the samples may lead to inaccurate responses by the dogs.

Similarly, geographical conditions may also have an impact on VOC-profiles (225). Chaber et al. showed slight differences in canine olfactory performance depending on the geographical origin of samples (117). However, special care should be taken to ensure that handling of utensils during sampling and processing proceeds in the same manner for both negative and positive samples across testing locations (47, 226, 227). In addition, Callewaert et al. (in preparation) found that dissimilarities in canine performance occur depending on the carrier material (e.g., cotton pad, cotton gauze, commercial odor carrier, etc.). Therefore, it is advised to use one and the same carrier throughout training and DTE.

For training and DTE purposes, a large representation of different demographic aspects (sex, age, etc.), localities of sample origin, and temporally different stages of infection among SARS-CoV-2-positive and -negative samples is crucial to adequately map the olfactory fingerprint of the disease. In addition, other aspects such as pre-existing infectious and non-infectious pathological conditions, recovered SARS-CoV-2-infections, Post-COVID-19 condition, COVID-19-vaccination status, or differing virus variants may play an important role for VOC-patterns and are subject of current research (51, 52, 69, 71). ten Hagen et al. studied the ability of dogs to discriminate between SARS-CoV-2-infections and other viral respiratory infections in NPS/OPS and infected cell cultures, when trained with saliva from SARS-CoV-2-positive individuals or with SARS-CoV-2-infected cell culture supernatants. Although sensitivity was lower (61.2–75.8%) than in other studies from the same laboratory, dogs rejected the samples of other viral infections in the DTE more often than SARS-CoV-2-infected samples, which is reflected by a high specificity of 90.2–95.1%. This indicates that further respiratory viral diseases defined as SARS-CoV-2-negative samples should always be integrated into training procedures in order to enhance diagnostic acuity for SARS-CoV-2 (41).

In terms of infection state, the crucial intervals dogs should be able to recognize is any phase in which viable virus is shed in order to contain the pandemic effectively. Therefore, samples across all phases of infection should be used for training in order to reliably indicate all potentially changing relevant odor-profiles in the course of infection. However, further research is needed to evaluate if and how dogs are able to transfer their olfactory detection abilities from a certain stage of disease to another. Recent studies found that dogs which were trained with samples from acute SARS-CoV-2-infection did not indicate patients with Post-COVID-19 condition as positive, when tested versus acute infection. Nevertheless, when tested against samples from healthy individuals, Post-COVID-19 condition samples were identified (51, 52). These results might suggest a titration effect, which could be based on a slow gradual decomposition of characteristic VOCs even if the virus is only residually or not present anymore.

Furthermore, an appropriate mapping of disease severity (e.g., asymptomatic, mild, severe) should be taken into account and integrated into training. However, COVID-19-VOC-measurement indicated that there was no relationship between VOCs and viral loads (34) or disease states, although mainly severe cases were included (36). Importantly, more research is needed to explore to what extent PCR-cycle threshold values, representing viral loads, influence canine olfactory performance [see also (47)].

Dogs can even be trained to certain concentration differences of the target scent (228). For example, this is used in diabetes alert dogs, which detect increases or decreases of blood glucose values of patients beyond predetermined levels (229). This emphasizes that the samples used in canine training procedures must be as versatile as possible. The issues described in this section will be minimized when further efforts are made in the profiling of critical SARS-CoV-2-VOCs and in the processing techniques of samples in order to reduce olfactory noise from potential exogenous and irrelevant endogenous factors (215). A crucial question which arises is whether training conditions can be reduced to the lowest common denominator by, for example, training with pure viral proteins or proteins produced in cell cultures or animal models (see section "Standardized sample alternatives"). Cell cultures were used in one (41) of the 27 reviewed studies by Meller et al. (4).

Pre-processing of samples

Many protocols for inactivation of viral pathogens with differing grades of loss of functional and structural viral integrity exist. The main purpose of viral inactivation in scent dog detection studies is the safe handling of training samples for animals and humans. On the other side, olfactory fingerprints of samples deriving from SARS-CoV-2-infections have to be preserved, probably requiring gentle inactivation methods. Different approaches up to renunciation of inactivation procedures were used in the reviewed COVID-19-scent dog literature (4), which is discussed below. The study from Jendrny et al. revealed that inactivated samples can be used for training to subsequently screen non-inactivated "armed" samples with a median sensitivity and specificity of 84 and 95%, respectively (134).

Beta-propiolactone

Beta-propiolactone (BPL) is an organic chemical compound which has historically been used for effective inactivation of various known viruses (230), especially in the field of vaccine development (231–234). BPL inactivates SARS-CoV-2 as well (235). Its inactivating properties are based on opening its lactone ring which is unstable in aqueous media and highly

reactive (230). Due to rapid hydrolyzation in aqueous media, the substance is transformed within a few hours to non-toxic 3-hydroxypropionic acid making it highly suitable and safe for biological preparations (236). Despite the rapid degradation, viral activity has usually subsided long before the last detectable residuals of BPL in samples have been measured (230). BPL appears to have affinity for viral nucleic acids blocking viral replication while mostly sparing the protein structures, which preserves the immunogenicity of the virus. However, not all the organic chemical modifications coming from BPL are elucidated and proteins may be affected as well (237). On the other hand, Determann and Joachim showed that a higher reactivity toward certain functional groups of amino acids results from a lower hydrolyzation capacity of the medium (238), highlighting that water is a preferred nucleophilic reagent of BPL. In summary, variations in nucleophilic characteristics of the reaction with BPL and the "nucleophilic potential" as well as further physicochemical properties of the medium might explain why varying quantitative and qualitative dynamics among reaction products from different organic compounds exist (237). In the study from Jendrny et al. dogs did not smell a relevant difference between BPL-inactivated (training) and non-inactivated (DTE) SARS-CoV-2-infected samples (134). Although more research is needed in this field, this might indicate that nucleophilicity of relevant VOCs is low and that microenvironmental aspects of the samples could further contribute to the lack of involvement of respective VOCs in the reaction with BPL so that those are kept preserved. Furthermore, it is possible that the BPL-manipulation has no effect on the high discriminatory power of the dog's olfactory system. It is noteworthy that in the first work by Jendrny et al., non-inactivated negative samples were used in addition to BPL-inactivated negative samples (133). The dogs did not indicate the latter more often than the former even though they were trained with BPL-inactivated positive samples (133). Only three of the reviewed studies used BPL for viral inactivation in their DTEs (41, 52, 133). However, in terms of safety versus VOC-preservation, BPL inactivation represents a highly effective and reasonable method.

Heat

Heat inactivation is a possible and common method to destroy viral pathogens effectively (239, 240). At the same time, maintenance of antigen integrity is important to preserve the diagnostic value of samples, e.g., for serological analysis (240–247). Heating methods can prevent infectivity of SARS-CoV-2 and at the same time preserve RNA when appropriate temperatures are applied (247). In contrast, BPL preserves proteins but not RNA (see above). Heat has denaturizing properties on proteins and other compounds leading to disruption in the interaction between virion and cell. Even slight alterations might also have a crucial and persistent impact on quality of the VOC-emitting properties of organic material, changing VOC-concentrations and their chemical composition. In addition, Lomonaco et al. showed that heat treatment is able to alter VOC-composition in human breath samples (220).

Heat inactivation or treatment in the DTEs was used in three (118, 145, 147) of the reviewed studies (4). Essler et al. (145) trained dogs with detergent-inactivated urine (see below) and tested the dogs for detection of heat-inactivated urine. Especially when dogs were confronted with a novel heat-inactivated sample, overall sensitivity was only 62%, whereas specificity was 98%. This may indicate that – at least in relation to detergent treatment – heat may alter critical COVID-19-VOC-profiles to a certain extent inducing uncertainty, or that the use of detergent inactivation made the odor more obvious. However, it has to be mentioned that these transfer trials consisted of only one set of presented samples to eight dogs. Interestingly, the performance of dogs, which were trained with heat-inactivated samples and tested with new heat-inactivated samples, deteriorated significantly, which possibly was due to a poor generalization process as sample availability was limited at the time the experiments were performed (145). Possibly, the process of heat-inactivation might produce different VOC-profiles among individual samples, depending on their original chemical and physical composition. The learned VOC-spectrum would thus present too broad to be finally used in detection of COVID-19-specific smell with adequate generalization and high diagnostic acuity. The assumption of global and individual changes in the key VOC-profile through heat-inactivation is also supported by the fact that the olfactory transfer performance from heat-inactivated urine-training to heat-inactivated saliva-testing produced very low sensitivities in two trials (11 and 22%, respectively), whereas the accurate recognition of negative samples was maintained (specificity of 94 and 100%, respectively) (145). However, only one positive sample was presented per trial across nine dogs. Furthermore, the discussed aspects of heat treatment remain speculative since other possible complicating factors have to be taken into account. In contrast, Jendrny et al. showed that dogs' transfer performance from BPL inactivated training samples to completely novel non-inactivated samples of the same and even different type is maintained at the same or even higher levels (134). BPL seems to retain the assumed global COVID-19-associated smell. Heat-inactivation appears to be more time-saving and cheaper than BPL-inactivation, but the former might lead to less robust learning results in dogs. Consistent with those statements, Salgirli et al. have also reported that dogs initially had problems recognizing heat-inactivated masks worn by COVID-19 patients when previously trained with non-inactivated masks (147). In contrast, Vesga et al., who used heat treatment in order to prevent proliferation of microbiota, reported a high performance quality of dogs, however, the treatment was not further specified (118).

Ultraviolet radiation

Ultraviolet-C (UV-C) radiation may be used to inactivate coronaviruses effectively (240, 248–250). It acts mainly by photochemical conversions of heterocyclic bases in the structure of nucleic acids without spontaneous reversion (251–253). Amino acids are affected to a lesser extent while carbohydrates and lipids are hardly modified (251). Mendel et al. used 10 minutes of UV-C-irradiation (254 nm) per side of mask material for SARS-CoV-2-positive cases, for canine training and for DTEs (146). Similarly, Salgirli et al. also used UV-inactivation (147). However, it is a significant concern of methodology in both studies that it is not clearly stated whether negative samples were also inactivated in order to control for potential pronounced or subtle UV-induced alterations in COVID-19-associated VOCs. In an additional experiment Mendel et al. showed that UV exposure did not result in statistically significant alterations in headspace solid phase microextraction GC-MS-profiles of at least 36 typical human-derived scent compounds pipetted on unused masks, suggesting a lack of significant photocatalytic effects on these VOCs (146). Conversely, UV-radiation of different wavelengths (especially UV-C) and dosage can have a great photocatalytic impact on gaseous emissions and VOCs by eliminating many of them from air samples, even within seconds (254–257), or from liquid media (258, 259). Therefore, the extent to which specific COVID-19-associated VOCs are altered by UV-irradiation remains uncertain and needs to be elucidated. If there are alterations, it has to also be clarified whether the discriminatory power of the canine olfactory system is nevertheless sufficient to compensate for those changes.

However, Mendel et al. (146) reported in two cases that trained dogs were able to indicate locations at workplaces where SARS-CoV-2-infected individuals had been situated 3–4 weeks prior to canine inspection. It would represent a promising indication that UV-irradiation might have no relevant effect on COVID-19-associated VOCs and that the temporal range of detection might extend well beyond acute infections, however, these are only few individual cases reported (146) and it remains questionable whether COVID-19-associated VOCs persist in a confined area for such a long time without appropriate storage [SARS-CoV-2 itself survives only a few days in the environment (260)]. In summary, comparative studies of training with UV-inactivated and DTEs with new, non-inactivated samples under high security standards (see also section "Susceptibility of dogs for SARS-CoV-2") are an essential step to ultimately verify the suitability of UV-inactivation for establishing canine training sample sets. Although the actual process of viral inactivation by UV takes longer than by BPL (230), the use of UV would be a time-saving and an ecological method since, in contrast to BPL- or detergent-inactivation, no chemicals, no targeted chemical manipulations of the samples, and no waiting time for hydrolysis are required.

Detergent–solvent

Detergent/solvent applications are a further method for efficient viral inactivation by complete destruction of the lipid membrane of enveloped viruses while preserving the structure of proteins from the virus and from the biological microenvironment (261–263). This method is widely and commercially used especially in the treatment of therapeutic human plasma, as it robustly destroys enveloped viruses while at the same time retaining physiological activity levels of plasma proteins (262, 264). Nonidet^{TM} NP-40 in combination with further detergents seems to successfully disrupt coronavirions (240) and was used by one canine COVID-19-detection study for urine inactivation (145). A possible VOC-alterating effect of Nonidet^{TM} NP-40 or the closely related substance Triton X-100 on VOCs in treated samples is not elucidated. They represent gentle inactivation methods, but it might be assumed that lytic effects on membranes of contained cells (265) might slightly change the biochemical properties of those samples. Triton X-100 has a vapor pressure of 130 Pa at 20°C and is therefore considered an organic volatile substance (266). It can therefore be assumed that the detergents themselves change the odor-profile of the samples while they are still dissolved. In order to clarify these issues, comparative canine olfaction studies with both detergent-inactivated and non-inactivated positive and negative samples are necessary. However, Essler et al. (145) could show that cognitive transfer from detergent-inactivated to heat-inactivated samples is possible. Although sensitivities decreased, this may also be due to heat-inactivation [(145); see also section "Heat"]. Finally, chemical treatments with detergents or BPL are more environmentally damaging, time-consuming, and eventually more expensive than, for example, the use of UV-C. Nevertheless, they appear to allow satisfactory and safe olfactory transfer to non-inactivated samples in canine COVID-19-detection (134, 145).

No viral inactivation

No inactivation can represent a biosafety issue, but is probably also the best method for the preservation of crucial VOC-profiles. SARS-CoV-2 can survive a few days in the environment depending on the type of contaminated surface (260). In secreted biological material like aerosols, virus was shown to be infectious for minutes to hours (260, 267) whereas other studies show higher viral activity up to 21 days in different body fluids like e.g., sputum, saliva, urine, and blood, depending on seasonal factors (268). No virus inactivation was used by the majority of reviewed canine COVID-19-detection studies in their DTEs (n = 21) (4). Sweat samples were the main material used without inactivation which *per se* have no high infectivity (see also section "Sweat and body odor"). Similarly, four studies did not use inactivation of mask or clothes samples (47, 144, 147, 190). Apparently, the material on which sweat or body odor was collected impacts the viral persistence as well, since cotton and related material seems to

ensure decomposition of its RNA within minutes (269, 270). The duration between sample acquisition and presentation to dogs was variable across studies between hours and months (see also section "Sweat and body odor"). However, in some reports, inactivation was omitted also in other sample types like saliva (184) and nasopharyngeal secretions (144) without use of further biosafety measures. Leaving out inactivation generated robust and good results (4) suggesting that characteristic VOCs outlast the virus presence or at least high viral loads. Nevertheless, independent of inactivation status, special safety measures should be used (118, 134, 145), e.g., Training Aid Delivery Device (TADD) containers (134, 145), which are supposed to allow odor particles to pass through but not droplet- or particle-bound virions, in order to protect both animals and humans (134). This is especially interesting for training purposes where body fluids with higher viral loads may be used. For detection of other infectious diseases, corresponding data about pathogen dynamics in body fluids and environment should be used, or reliable data should be generated first in the case of future emerging zoonotic diseases in order to determine susceptibility of dogs to pathogens of interest and to guarantee adequate safety.

Standardized sample alternatives

Training sample sets derived from naturally obtained human biological fluids can be used for extended periods of time. However, more research is needed in appropriate storage conditions (see section "Sample types"). In addition, acquiring samples is not trivial both from an ethical and logistic sense, especially early in a pandemic or while pandemic dynamics are low. Furthermore, storage duration and divergent storage conditions can affect VOC-patterns (221, 271, 272). There may also exist uncertainties regarding true infection status with possible false negative or false positive PCR-status potentially corrupting the sensitive training process for the right olfactory cue, differences in temporal and clinical infection states, demographic differences, etc.

Producing specific COVID-19-associated VOC-profiles artificially for dog training purposes represents a challenging endeavor although first approaches with a "VOC-cocktail" have been conducted in combination with eNoses (273). However, sensor array composition of the eNose and environmental influences still represent a major limitation and studies are merely scratching the surface of decoding the volatilome of SARS-CoV-2-infections (see section "The smell of COVID-19"). For the current state of the canine COVID-19-detection research, it was important to cover the majority of VOC-variations, which can emerge from varying disease-associated factors, for adequate broad training and generalization. Nevertheless, it is only the attempt to "catch" the true critical COVID-19-odor of an active infection in an as broad as possible way, for the simple reason that the critical VOC-composition is not known yet but at the same time early investigation of anti-pandemic measures appeared reasonable. In this way, dogs were taught to perceive key signals from a broad array of positive samples, which were not present in a broad array of negative samples. Therefore, dogs did not learn to detect an absolute COVID-19-VOC-profile, but a certain scent-profile of relative difference to what healthy individuals did not express.

The VOC-hypothesis is based on viable metabolic entities with the result of VOC-production, which may have fingerprint-like properties for certain pathological conditions, e.g., viral infections. Some have suggested that dogs might be able to detect viral proteins, i.e., spike proteins (51, 52), which could be perceived by olfaction without any metabolic intermediate step. Amino acids are not among the substances typically defined as odorants, and to date have been little studied in the context of odor perception, except in fish (274–278). Humans have been shown to be able to distinguish among certain amino acids by olfaction (279, 280). Whether dogs are able to smell parts of the pure SARS-CoV-2-proteins and reliably discriminate it against other distractors is currently being investigated. In this context, the function of the vomeronasal organ in dogs should be emphasized, which serves as an additional olfactory organ for intra-species communication through pheromones and is located rostrally at the bottom of the nasal cavity (5). Interestingly, in contrast to the main olfactory organ, the vomeronasal organ is capable of detecting non-volatile molecules of higher molecular weight, such as proteins (281), which might indicate the presence of different receptor cell types in both olfactory organs (282).

If dogs are able to smell viral proteins, a standardized, broad, and sustainable training infrastructure based on appropriately manufactured proteins could be established and research is already underway. Safety would be guaranteed due to the absence of the viable virus, however, the risk of contamination of such sensitive samples is high. In addition, costs of sampling body fluids versus production of protein samples must be considered. An essential consideration, however, is the periodic emergence of new variants of SARS-CoV-2 with differing mutations in spike genes and protein expression (283, 284). When dogs are trained on a single protein the spectrum of detection would be extremely narrow and certainly highly specific, increasing olfactory discrimination (129), but it has to be studied whether it would suffice to cover different virus variants. It would therefore seem reasonable to mix the variants during training sessions according to the current viral occurrence in the population. However, it may take some time before the next corresponding protein is available after discovery and identification of a new variant.

It is probable that the impact of viral variants on variation in COVID-19-VOC-patterns is less pronounced. Chaber et al. stated that dogs had no difficulties recognizing the virus despite being confronted with different strains in biological samples

(117). On the contrary, Kantele et al. showed a significant difference in accuracy between variants, when the training included only biological samples with the wild-type virus (69). Furthermore, by using only proteins for training, it would be essential that viral material is present and "readily accessible" in the screening samples of diseased individuals, which is not always the case for sweat/body odor or urine as described above (see section "Sample types"). In contrast, only infected individuals who acutely excrete the virus would be detected in this way sparing individuals who do not shed the virus anymore but still express COVID-19-VOC-patterns. Nevertheless, this can be deceptive because the viral load may vary temporally across the samples while the individual is still infectious or may depend on vaccine-induced immune response (see section "Sample types").

In order to circumvent these issues, supernatants from infected human cell cultures (41) could be used for additional training with negative/distractor samples belonging to the same culture or with different cell lines among positive and negative samples, profiting from the advantages of VOCs. The combinatorial approach of VOCs and proteins (e.g., one or more "sets" of specifically trained dogs, see section "Olfactory generalization") could maintain high levels of sensitivity in general screening and be used in special confirmatory cases as a highly specific detection method for certain dangerous viral variants, which could be continuously updated in dogs' olfactory memory (117).

Although cell cultures are a very interesting alternative, it should be noted that the VOC-profile does not necessarily correspond to the versatility of VOC-patterns from naturally obtained biological samples. Murarka et al. trained dogs with an ovarian cancer cell line and showed that olfactory transfer or switch from cell culture to samples of patients with ovarian cancer did not readily occur in dogs (285). Similarly, there was a lower detection ability in SARS-CoV-2-positive cell culture supernatants after dogs had been trained with naturally acquired saliva samples (41). These problems could be circumvented to some extent by using different cell lines in cell cultures, but mimicking the olfactory versatility of naturally acquired samples remains difficult. Another alternative may be the use of SARS-CoV-2-training samples from animal models. Nevertheless, the question of effective translation to human derived VOCs needs to be addressed (286). Despite the great advantages of sample alternatives with regard to trainability and standardization, the use of "real" biological samples will probably still be necessary to prepare dogs for real-life screening scenarios.

Target and screening population and the operational applicability

The World Health Organization (WHO) and the German Paul Ehrlich Institute (PEI) recommend thresholds for diagnostic sensitivities and specificities for point-of-care-antigen tests to be more than 80% and more than 97%, respectively (287). 78% of reviewed canine detection studies showed $\geq$ 80% sensitivity and 60% of studies showed $\geq$ 95% specificity. Therefore, dogs' detection is in line with or even better than other rapid diagnostic tests. Dogs achieved even better performances when only considering high-quality studies with a low risk of bias (4).

However, when considering the entire components influencing the dog as a detection system, it has to be taken into account that the characteristics of the population to be tested has its impact on the accuracy as well. Besides the actual prevalence of COVID-19 within the target population to be screened the detection performance differs between different populations and search scenarios, which has a direct impact on the practicality. Therefore, the calculation of expected positive and negative predictive values is crucial for the decision on screening scenarios in order to avoid any vilification of the dogs' detection (**Table 2**).

It is furthermore important to note that not all studies relied on a single dog's decision to determine sensitivity and specificity. In particular, in some cross-sectional studies, decisions from multiple dogs were used to ensure certainty in defining the infection/disease-status of tested individuals (69, 71, 191, 194). Those considerations, which also might depend on the number of available trained dogs, are important especially for the planning and conduction of a screening test. Furthermore, changing and distracting environmental factors should be reduced or avoided in the operational screening setting (see also section "Dog operational environment").

TABLE 2 Positive and negative predictive values for dogs' performance of 90% sensitivity and 99% specificity and for the recommendations of the World Health Organization (WHO) and Paul Ehrlich Institute (PEI) among different COVID-19 prevalences in the target population.

COVID-19 prevalence	Dogs' performance (SEN = 0.90, SPE = 0.99)		WHO and PEI recommendations (SEN = 0.80, SPE = 0.97)	
	PPV	NPV	PPV	NPV
0.0010	0.0826	0.9999	0.0260	0.9998
0.0011	0.0902	0.9999	0.0285	0.9998
0.0012	0.0976	0.9999	0.0310	0.9998
0.0013	0.1049	0.9999	0.0335	0.9997
0.0014	0.1120	0.9999	0.0360	0.9997
0.0015	0.1191	0.9998	0.0385	0.9997
0.0016	0.1261	0.9998	0.0410	0.9997
0.0017	0.1329	0.9998	0.0434	0.9996
0.0018	0.1396	0.9998	0.0459	0.9996
0.0019	0.1463	0.9998	0.0483	0.9996
0.0020	0.1528	0.9998	0.0507	0.9996

SEN, sensitivity; SPE, specificity; PPV, positive predictive value; NPV, negative predictive value.

Dog detection as the one health approach to tackle COVID-19

The increase of zoonotic infectious diseases highlights the importance of collaborative, multisectoral and interdisciplinary work to address challenges that could impact public health, animal health and production, and environmental conservation. The World Health Organization (WHO), the Food and Agriculture Organization of the United Nations (FAO), the World Organization for Animal Health (WOAH) and the United Nations Environment Programme (UNEP) have established an intersectoral collaboration aiming to implement initiatives under the concept of "One Health" to address main global problems at the human-animal-environment interface (288). The four organizations are working together to mainstream One Health so that they are better prepared to prevent, predict, detect, and respond to global health threats and promote sustainable development. The use of COVID-19-detecting dogs is a great example of using this concept to respond to the current COVID-19-pandemic. In this review, we showed how multi-sectoral communication and joint work resulted in the generation of evidence that the use of dogs trained to detect SARS-CoV-2-infections has been shown to be a rapid, mobile, and non-invasive tool for early detection of affected individuals. Collaborative efforts are crucial to minimize the rapid viral transmission requiring massive testing (289, 290). The use of detection dogs to pre-screen infections among the population could overcome the overloaded response capacity of laboratories due to the higher number of required tests, the lack of needed reagents to perform these tests, and technical issues in sampling infected individuals (i.e., inappropriate sample collection, storage, or transportation) or false-negative results related to the disease status with low viral multiplication levels (291–293).

Conclusion

Dogs can detect samples from SARS-CoV-2-infected individuals with a high degree of diagnostic accuracy. However, the search context, study design and quality of the current studies varied considerably, and only a small percentage of studies were of high quality with a low risk of bias. In contrast to an industrially produced test kit, dogs and their olfactory performance are naturally subject to many variations. In addition, disease detection involves difficult to measure and volatile amounts of substances and little is known about the olfactory dynamics of a pathological process, making it difficult to control the process of adequate odor imprinting. However, the evidence of canine COVID-19 recognition has been replicated by several different groups, and the dog proved to be an incomparably fast detection tool. Importantly, in epi/pandemic conditions, dogs can be trained quickly with a good level of sensitivity before specific laboratory methods are available, helping with isolation of infected patients presenting with or without symptoms. Therefore, further research on influences of the odor profile (or the perception of it) by factors such as training sample number and type, sampling method, inactivation type, training procedures, dogs' personalities, environment, translation from training to test scenario, etc. proves to be very important for harmonization and optimization of canine scent detection and for maintenance of high study quality. Those considerations pave the way for the canine olfaction to become a reliable, stable and quick test method. Thus, standardization and validation processes such as those used in the field of drug and explosive detection dogs are urgently needed, if medical detection dogs should be deployed in the field to detect samples from SARS-CoV-2-infected individuals.

We recommend the use of dogs as VOC-detectors in mass screenings as a quick, highly adaptive, and effective countermeasure both at the emergence and also in the further course of a pandemic, provided that sufficient numbers of diverse positive and negative, high quality, safe samples for training purposes can be generated early, the pathophysiological condition of those samples is known with a high certainty, and that training procedures, dogs, and their handlers are certified similarly as described for scent detection in explosives (294).

Author contributions

HV and SM discussed and planned the form of the comprehensive analysis. SM wrote the manuscript and conducted the main literature research. All other authors have contributed significantly to the completion and development of the review and provided their expert opinion. All authors have read and approved the final manuscript.

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Conflict of interest

JGL was employed by the Arctech Innovation. HE and JE were employed by the Kynoscience UG.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.1015620/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Detailed mind map representing areas of interest and related aspects that played a role in the reviewed canine COVID-19-detection studies by Meller et al. (4) and have been highlighted by the experts in this publication.

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Sniffing out safety: canine detection and identification of SARS-CoV-2 infection from armpit sweat

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Detection dogs were trained to detect SARS-CoV-2 infection based on armpit sweat odor. Sweat samples were collected using cotton pads under the armpits of negative and positive human patients, confirmed by qPCR, for periods of 15–30 min. Multiple hospitals and organizations throughout Belgium participated in this study. The sweat samples were stored at −20°C prior to being used for training purposes. Six dogs were trained under controlled atmosphere conditions for 2–3 months. After training, a 7-day validation period was conducted to assess the dogs' performances. The detection dogs exhibited an overall sensitivity of 81%, specificity of 98%, and an accuracy of 95%. After validation, training continued for 3 months, during which the dogs' performances remained the same. Gas chromatography/mass spectrometry (GC/MS) analysis revealed a unique sweat

scent associated with SARS-CoV-2 positive sweat samples. This scent consisted of a wide variety of volatiles, including breakdown compounds of antiviral fatty acids, skin proteins and neurotransmitters/hormones. An acceptability survey conducted in Belgium demonstrated an overall high acceptability and enthusiasm toward the use of detection dogs for SARS-CoV-2 detection. Compared to qPCR and previous canine studies, the detection dogs have good performances in detecting SARS-CoV-2 infection in humans, using frozen sweat samples from the armpits. As a result, they can be used as an accurate pre-screening tool in various field settings alongside the PCR test.

KEYWORDS

COVID-19, detection dogs, GC/MS (gas chromatograph/mass spectrometry), acceptability analysis, odor, axilla, vaccination

Introduction

The SARS-CoV-2 pandemic had dramatic economic and social consequences on a global scale. There was a need for a fast, reliable, inexpensive, easy, non-invasive and widely applicable screening method to distinguish SARS-CoV-2 carriers from non-carriers. Rapid screening and identification of symptomatic as well as asymptomatic and presymptomatic people can contribute to reducing the basic reproduction number of the virus. Even with vaccination efforts, the necessity for fast and reliable detection tools remains crucial to avoid new outbreaks.

Current diagnostic tests are time-consuming, and often come with a considerable cost, while nasopharyngeal swabs are semi-invasive. The predominant method of virus identification, quantitative polymerase chain reaction (qPCR), entails significant time, effort and expense to obtain results. Moreover, the qPCR test gives a considerable amount of false negative results (ranging from 2 to 29%) (1, 2) primarily due to the inherent instability of viral RNA and the potential inadequacy of nasopharyngeal or oral samples in providing enough material. Distinguishing asymptomatic carriers from uninfected individuals can also pose challenges. Additionally, temperature-based screening methods, such as automated forehead temperature sampling, only detect symptomatic people and are inadequate for comprehensive screening purposes.

The human body reacts to the viral infection by producing white blood cells and immune factors. This immune response results in the secretion of various biological molecules and immune factors, some of which are excreted through the skin. Among the regions of the body where these immune factors are notably concentrated is the apocrine sweat regions, particularly the armpits (3). The armpits likewise harbor important lymph nodes that contribute to the production of immune factors. Any bacterial, viral or fungal infection is associated with a unique volatile organic compound (VOC) creation from human cells. These VOCs are subsequently excreted through sweat and are an easy target for rapid screening purposes (4).

Dogs have an extraordinary olfactory capacity and have been successfully used to detect narcotics, explosives, cancer, malaria, metabolic diseases and a wide variety of bacterial and virus infections (5–10). Their trainability using positive reinforcement methods makes them well-suited for detection tasks (11). Common breeds of detection dogs are Belgian Malinois Shepherds, German Shepherds, Cocker Spaniels, Springer Spaniels, Labradors, Pointers, Border Collies, and Beagles. Notably, detection dogs have been employed in diverse settings such as airports, (large) companies, healthcare institutions (hospitals, retirement homes, and triage centers), shopping centers and various mass events including sporting events, cultural gatherings, fairs, concerts and festivals.

Detection dogs are trained to detect SARS-CoV-2 infection based on sweat odor. Previous studies have presented evidence that dogs are able to discriminate SARS-CoV-2 positive from negative samples (12, 13). Preliminary findings show that the dogs were able to discriminate between saliva samples of infected and non-infected individuals with average diagnostic sensitivity of 83% and specificity of 96% (13). Similar preliminary findings, using armpit sweat samples, showed that dogs were able to discriminate with a sensitivity of 83–95% (in 4 dogs) and up to 100% (in another 4 dogs) (12). These encouraging findings form the basis for the development of a reliable screening method for identifying SARS-CoV-2 infected people through the utilization of trained detection dogs.

SARS-CoV-2 detector dogs were trained in many countries around the world, including the United Arab Emirates (UAE), Lebanon, Australia, Chile, Argentina, Brazil, Germany, UK, Finland, France, USA, Russia, Italy, Spain, Colombia, Mexico, Poland, Iran, Peru, Czech Republic, Romania, Canada, Philippines, Switzerland, Saudi Arabia, Austria, Sweden, Georgia, Egypt, Honduras, Tunisia, Bahrain, Singapore, El Salvador and Belgium. A first proof of principle was recently obtained in France (using axillary sweat), Germany (using saliva) and in Finland (using urine). Dogs were trained and could distinguish with a very high success rate positive from negative samples, some of them with up to 100% accuracy (12, 14). SARS-CoV-2 dogs have been deployed in airports and borders in UAE, Lebanon, Saudi Arabia, France, and Finland.

The primary objectives of the present study were as follows:

- To establish a comprehensive biobank consisting of a large sample pool, which would be accessible to all relevant actors in Belgium involved in the training detection dogs. This would facilitate efficient training of the dogs, and ensure the availability of sufficient material for future upscaling.
- To develop a field-testing protocol to train detection dogs in distinguishing between samples infected with SARS-CoV-2 and

those that are not infected. This protocol aimed to optimize the dogs' detection capabilities in real-life scenarios.
- To identify the specific VOCs that the detection dogs are detecting and identify which volatiles make up the characteristic scent observed in SARS-CoV-2 positive sweat samples.

Materials and methods

Clinical trials

The protocol has received the approval of Animal Ethical Committee (ULiege, N°20-2246), as well as the approval of the Ethical Committees (Comité d'Ethique Hospitalo-Facultaire University of Liège, approval number 2020/139; Ethical Committee UZ Gent, approval number multicentric study BC-08571, coupled to CHU St Pierre Brussels, AZ Glorieux Ronse (study number TC20/12), AZ Oudenaarde, OLV Hospital Aalst, Jan Yperman hospital Ieper, ZNA hospital (study number 5491), GZA hospitals (study number 210304ACADEM), Jan Palfijn hospital Ghent, AZ Maria Middelares Gent (study number MMS.2021.006), AZ Sint-Vincentius Deinze (study number MMS.2021.006), AZ Jan Portaels Vilvoorde (study number 2021-01), AZ Sint-Lucas Ghent (study number 2020-32), WZC Curando Ruiselede, WZC Armonea, Hospital Saint-Pierre Ottignies) of the different hospitals collaborating to the study. Sweat donors (patients and healthy people) also signed an informed consent at sampling.

Sweat samples

From October 2020 until April 2021, sampling was organized in Belgium in different hospitals. Positive samples came from CHU-Liege, CHU-ND-Bruyeres, CHU St-Pierre Brussels, St-Pierre Ottignies, UZ Gent, AZ Glorieux Ronse, AZ Oudenaarde, OLVZ Aalst, Jan Yperman Ieper, UZA Antwerp, ZNA Stuivenberg, GZA Anvers, AZ Klina Brasschaat, Jan Palfijn Gent, AZ St-Vincentius Deinze, St-Trudo St-Truiden, AZ Jan Portaels Vilvoorde, AZ Alma Eeklo. Negative samples came from the Kiwanis organization, who organized sampling in different cities in Belgium. Additionally, different care centers (hospitals, senior homes) organized sampling: WZC Armonea Wilrijk, WZC Armonea Spanjeberg, Zorg-Saam WZC Oostakker, WZC Curando Ruiselede, CHU-Liege, CHU St-Pierre Brussels. A list of metadata was collected from each patient/participant, including date, age, biological gender, weight, height, Body Mass Index, ethnicity, postal code, deodorant use, deodorant use frequency, hygiene habits, frequency of underarm washing, medication use, hormonal contraception use, antibiotics use, smoking, comorbidities, (hospital) location of sampling, SARS-CoV-2 symptoms, Ct-value of qPCR result. In the essence of time, dogs used in the present study were trained on a large and diverse set of samples including different hospitals/elderly homes, young and old people, male and female persons, smoker and non-smoker; deodorant user and no underarm cosmetic users.

All sweat donors had their SARS-CoV-2 status (negative or positive) confirmed by qPCR. For positive samples, only patients with clear symptoms (hospitalized) and qPCR results of <30 cycles were preferred. Patients were tested multiple times in the hospital. Patients with no PCR-confirmed test and/or vaccinated against SARS-CoV-2 were excluded. Patients in hospitals with clinical signs related to SARS-CoV-2 (respiratory symptoms, fever) but negative (qPCR) to SARS-CoV-2 were also included. With each donor, a complete but anonymized clinical metadata file was completed. The sweat sampling was performed by trained doctors and/or nurses for safety reasons. It consisted of 5 cotton balls or sterile compresses placed under the 2 armpits of the patient/donor during 15–30 min. For a subset of patients, the sampling was repeated on different days, as long as patients were still SARS-CoV-2 positive. The sampler wore nitrile gloves and a coverall (biological hazard) and handled the samples with a clamp, before putting them in a glass jar or in a closed plastic bag (ziplock). Within 1 h, the plastic bag or the glass jar were frozen (−20°C or colder) and stored until the training of the dogs. Temperature inside the freezer was constantly recorded and was found to be stable.

The tested samples during validation and post-validation were obtained from the original SARS-CoV-2 virus (WIV04 / 2019). At a later stage, samples were obtained from vaccinated people at CHU Saint-Pierre Brussels at least 3 weeks after the second dose of their vaccine (Comirnaty, BioNTech-Pfizer). These samples were also presented to the dogs, together with a positive control sample.

Dog selection

The dogs were Malinois Shepherds, Border Collie and Springer Spaniel from Federal Police, Civil Security and Army, with previous functions as explosive detection and urban search and rescue. Six dogs were enrolled in the present study up to the validation phase:
- Lilly, Springer spaniel, female, 3 years-old, explosives detector dog, Army
- Xhena, Malinois Shepherd, female, 7 years-old, explosives detector dog, Army
- Tina, Malinois Shepherd, female, 3 years-old, explosives detector dog, Army
- Paxy, Border collie, female, 4 years-old, search and rescue dog, Civil security
- Bailey, Malinois Shepherd, female, 1 year-old, explosives detector dog, Federal Police
- Chaeos, Malinois Shepherd, male, 1 year-old, explosives detector dog, Federal Police.

Dog training

The training took place in Neerhespen (Belgium) at the dog training and accreditation center of the Federal Police (DACH). A spacious room (10 m × 8 m, 80 m^2) with permanently air-conditioned controlled temperature (16°C) and relative humidity (30%) was used during the training. Metal cones (stainless steel), about 50 cm high from the ground, were used to release the smell of the sweat odors. Behind these cones, a glass jar containing the sweat sample was screwed on and placed in a larger metal box. There was no direct contact between the dog's nose and the sweat sample (Figure 1).

Sweat samples were removed from the freezer at least 30 min prior to their use. Two cotton balls/compress from one patient

FIGURE 1
Detection dog (Malinois shepherd) sniffing a sweat sample through a metallic cone during a training at the Training Center of Neerhespen (Federal Police, DACH).

were placed into the metal box behind the cones for each dog. The same human patient (2 × 5 cotton balls/compress) could be tested by 2–4 different dogs (2–3 cotton balls per dog per run). After each run (detection of 6–10 samples in a line), the metal cones were wiped off by a cloth soaked in water with 3% of acetone.

Training of the dogs was based on positive reinforcement and classical and operational conditioning principles using primary and secondary reinforcers. When the dog indicates a correct positive sample, a clicker is used to reward the dog. After the click the dog receives a toy (secondary) or food (primary reinforcer). On a negative run the dog gets his toy when there is no false positive indication.

The training was organized in four different steps, beginning mid-December 2020 and ending at the beginning of March 2021 (including validation), for a total of 10 weeks:

1. Odor fixation. On one single cone, dogs sniffed only positive samples of different origins in order to learn how to mark the samples (the dog sits, lies down and/or remains motionless in front of the positive sample). This part lasted 2 weeks.
2. Inclusion of blank samples next to positive samples. Blank samples are compress/cotton balls without sweat. Several cones involved. This part lasted 1 week with about 3 to 4 runs per dog per day.
3. Inclusion of negative samples next to blank and positive samples. Six cones included in the training. This part lasted 2 weeks with about 4 runs per dog per day.
4. Only positive and negative samples, no blank. Six to ten cones were presented in a line to the dogs. This part lasted 3 to 4 weeks with about 4 to 6 runs per dog per day.

After the training, a week (7 days) of validation was organized in the dog center of Neerhespen. This validation was performed in double-blinded conditions whereby neither the dog/handler, nor the second person with the clicker was aware of the number and/or the position of positive samples. The runs consisted of 6 metal cones in line containing either all negative samples, or negative and positive samples (1, 2, or 3 positive samples and the rest negative), but without blank samples. The number of positive and negative samples, as well as the order in the line was randomly attributed for each dog. A same positive sample was systematically tested by 2 dogs, in order to detect any trouble regarding the quality of the sample. The performances of the dogs were calculated after the validation process.

After the validation phase, training continued for 4 more months. Performances after this post-validation phase were also measured and compared to validations' performances.

GC/MS analysis and GNPS identification

60 SARS-CoV-2-positive sweat samples, 60 SARS-CoV-2-negative sweat samples and 14 blank samples were analyzed using GC/MS to identify the volatiles present in the sweat samples. The GC/MS analysis was carried out using the Agilent 7200 GC QTOF Agilent Technologies Santa Clara (CA) equipped with a robotic sampler system. The separation was conducted on an HP 5MS column (30 m × 0.25 mm × 0.25 μm). The patch within the vial was heated for 25 min at 200°C to desorb volatiles from the patch and 0.5 mL of headspace injected (injector temperature set at 250°C) into the instrument with a headspace syringe heated to 145°C. The GC protocol analysis included starting temperature 45°C min oven ramp (hold of 2 min), 15°C per min oven ramp to 325°C (hold of 3 min),

and 50°C per min oven ramp to 325°C purge the column for reaching equilibration it was 0.2 min for each cycle. The parameters of headspace; Oven, Loop, and Transfer line temperatures were 200°C. Timing parameters for headspacer were 3 min for Vial Equilibration, 0.2 min for Vial Pressurization, and Loop fill, 0.05 min for Loop Equilibration, and 0.2 min for injection. The helium carrier gas was set to constant 1.2 mL per min flow and a splitless injection mode was applied. The purge flow to split vent rate was 50 mL per min at 1 min. The collision gas was N_2 and collision flow was 1.5 mL per min. Also, the pressure was 9.466 psi and vial pressure was 10 psi. The He gas was used as a quench and Aux gas. The scanned m/z range was 35–400 with the acquisition rate of 10 spectra per second. The empty vial blanks were interspersed with the samples to assess the background signal. Features dataset was normalized. Features dataset were filtered and volatiles present in blank samples were subtracted and removed. Deconvolution and identification of GC/MS spectra was done as described before (15) and using GNPS (16). A PLS DA plot was constructed to understand the distribution of SARS-CoV-2-positive versus SARS-CoV-2-negative samples. Pairwise correlation analyses and random forest analyses were performed to understand differences between positive and negative sweat samples.

Statistical analysis

This study was held according to the STARD 2015 guidelines (Standards for Reporting Diagnostic Accuracy Studies) (17). The PCR-test was considered in the present study as the "gold-standard" to which our dogs were compared. Sensitivity, Specificity, Accuracy, Positive predictive value, Negative predictive value and Youden index were calculated for each dog, after the validation week and after the post-validation training, using the original formulas (18). The positive and negative samples, as well as the dogs, were randomized beforehand.

Survey/questionnaire

An online national survey was set up to investigate the overall acceptability toward the use of SARS-CoV-2 detection dogs in practice and distributed through social media, the university websites of UGent and ULiège and through the national press toward the Belgian population. The survey was set up in French and in Dutch. The survey ran from March 5, 2021 till April 19, 2021, with the large bulk obtained in the first week. About 3,591 participants filled in the survey completely. Inclusion and exclusion criteria were residence (only inhabitants of Belgium), language (proficient in Dutch and French). No participants were excluded based on age, sex, race or ethnicity. About 63% of the Belgian population spoke Dutch and 37% spoke French, representing the different language groups in Belgium (Dutch in Flanders, French in Wallonia and both in Brussels). 43.6% of the participants were aged 21-40y, 45.9% were aged 41-65y, 5.4% were younger than 21y and 5.0% were older than 65y. Three quarters of the participants were female (75.3% – or 2,704 of the 3,591) and one quarter was male. Results were combined and translated to English and analyzed in R. To identify main correlations between results of questions, correlation analyses and random forest analyses were performed.

Results

Sampling and optimal training protocol

The present study used the standard protocol developed in France as foundation for its research (12). The implementation of this protocol required adjustments due to encountered challenges. Initially, 15 detection dogs from different organizations, specialized in detecting explosives and human search tasks, were included in the study. However, not all dogs were suitable for this study, leading to a reduction of participating dogs. Some of the Urban Search & Rescue dogs were excluded due to difficulties encountered when working with cones positioned in line. The availability of sufficient positive and negative sweat samples was also crucial to allow an in-depth high frequency training of the dogs. The training regimen required several runs per day, with each dog undergoing up to 300 and more runs for a complete training. Initially, reuse of samples was done but was abandoned to avoid undesirable imprinting of specific samples in the dogs. The number of dogs involved in the study was reduced to 6, in order to increase the frequency of runs from 3 to 6 runs per day. A 5 day per week schedule was followed. The availability of a substantial number of positive and negative sweat samples was crucial, considering the high turnover of these samples. Additionally, efforts were made to improve the cleaning process of the cones between runs as well as to enhance the practical organization of the runs, including a correct recording of all results. These adjustments aimed to optimize the training process and manage the practical aspects of the study effectively.

Dog training results during and after validation

The validation process involved the utilization of 397 positive samples from 51 different patients and 1,629 negative samples collected from 276 volunteers. Each dog underwent the task of detecting an average of 66 ± 11 (Mean ± SD) positive samples and 272 ± 28 negative samples during the validation phase. This consisted a total of 58 ± 6 runs per dog over a 7-day period. The individual presentation of samples per dog as well as the Sensitivity, Specificity, Accuracy, Positive predictive value, Negative predictive value and Youden index of each dog after validation are represented in Table 1. Further individual details of the sample detection by the dogs can be found in Supplementary Table S1. The combined performance of all dogs yielded an overall specificity of 98% and an overall sensitivity of 81%. The overall accuracy amounted to 95%. The performance of the test, all dogs combined, evaluated with the Youden index was almost 80%.

The 6 same dogs were involved in continued training after the validation phase for 4 months (approximately one training every 2 weeks), from mid-March 2021 until the end of May 2021. They went to 6 training sessions of one-day each, with a total of 48 ± 6 runs per dog, and thus an average of 9 ± 2 runs per dog per training. The dogs were confronted to about 396 negative samples and 46 positive samples. During the post-validation phase, the dogs had to test different patterns of cone distribution: 5 to 10 negatives with all negatives, or a combination of 1 to 3 positive with 5 to 10 negatives. Table 2 summarizes the performances of the 6 dogs after the

TABLE 1 Diagnostic performances of the six detection dogs after validation.

Dog	Se* %	Sp* %	PPV* %	NPV* %	Youden %	Accuracy %	N Run*
Paxi	88	100	100	97	88	98	51
Cheos	81	99	95	95	80	95	61
Xhena	94	96	83	99	90	96	50
Tina	73	95	78	94	68	91	56
Lilly	76	100	100	94	76	95	63
Bailey	76	100	98	95	76	95	65
Total	81	98	92	95	79	95	58 ± 6

*Se, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value; N, number.

TABLE 2 Performances of the six dogs at the end of the post-validation phase (six trainings).

Dog	Se* %	Sp* %	PPV* %	NPV* %	Youden %	Accuracy %	N* Run
Paxi	88	100	97	98	88	98	49
Cheos	96	99	93	99	95	99	51
Xhena	78	99	96	96	78	96	58
Tina	76	98	85	96	74	95	44
Lilly	73	100	100	95	73	96	40
Bailey	70	100	100	95	70	96	46
Total	80	99	95	97	79	97	48 ± 6

*Se, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value; N, number.

post-validation phase. The overall specificity and sensitivity were 99 and 80%, respectively. The performance of the test (Youden index) was almost 80%. The performances of the dogs at validation and after validation are not significantly different ($p > 0.1$).

Dog results on vaccinated patients' samples

Sweat samples were obtained from 28 vaccinated people, who received the Comirnaty vaccine (BioNTech-Pfizer), in two doses and were sampled (armpit perspiration, cotton pads frozen at −20°C before training) 3 weeks after the second dose. These samples were tested by five dogs during training. The vaccinated samples were mixed along with other negative (unvaccinated) and positive (SARS-CoV-2) samples (each run included at least 6 different samples). Each dog performed 2 ± 1 runs (minimum 1 run, maximum 4 runs). The overall performances showed Se 77%, Sp 100%, and Youden index 0.77 (Supplementary Table S3). When taking into account only the vaccinated samples, the dogs considered the samples as negative in 100% of the cases.

Effect of age, biological gender, body mass index, deodorant use, medication use and sample location on detection by the dogs

Sampling location, and more importantly, sampling time were important influencing factors in the detection rate by the detection dogs. We found significant differences in marking by the detection dogs based on sampling location and sampling time (Figure 2). A shorter sampling time resulted in a lower detection rate by the detection dogs. Of the 13 samples that were taken from SARS-CoV-2-positive patients in hospitals that were held for only 15 min, instead of 30 min, only 1 sample was marked as positive by all six detection dogs. A sampling time of 30 min resulted in a significantly higher detection rate by the dogs ($p = 0.0031$). A shorter sampling time of 15 min was employed at CHU St-Pierre in Brussels and CHU Sart-Tilman Liège. The percentage of marking by the detection dogs was lower for these two hospitals ($p = 0.0078$ for CHU Liège as compared to Hospital OLV Aalst, where sampling was done for 30 min). The Saint-Pierre hospital in Ottignies also showed lower detection rates as compared to OLV Aalst ($p = 0.034$). A different sampling method or incomplete understanding of the sampling protocol could also affect the detection rate by the dogs.

There was no significant correlation found between the age of the participants providing the samples and the dogs' detection (or hesitation), although the age of the participants significantly differed between SARS-CoV-2 positive and negative samples (Supplementary Figure S1). The biological gender of the patients or volunteers did not influence the marking or hesitation by the six trained SARS-CoV-2 detection dogs, in either the SARS-CoV-2 positive or negative group (Supplementary Figure S2). A series of other variables were tested on their potential correlation with the detection rate by the detection dogs. Body mass index (BMI) had no influence on the marking by the six trained detection dogs during the validation phase (Supplementary Figure S3). The BMI was comparable among the SARS-CoV-2 positive and negative samples. Deodorant use similarly did not impact the marking by the six trained detection dogs during the validation phase. No significant differences were found between samples coming from people that either did or did not use deodorant. Medication use by patients or volunteers was tested

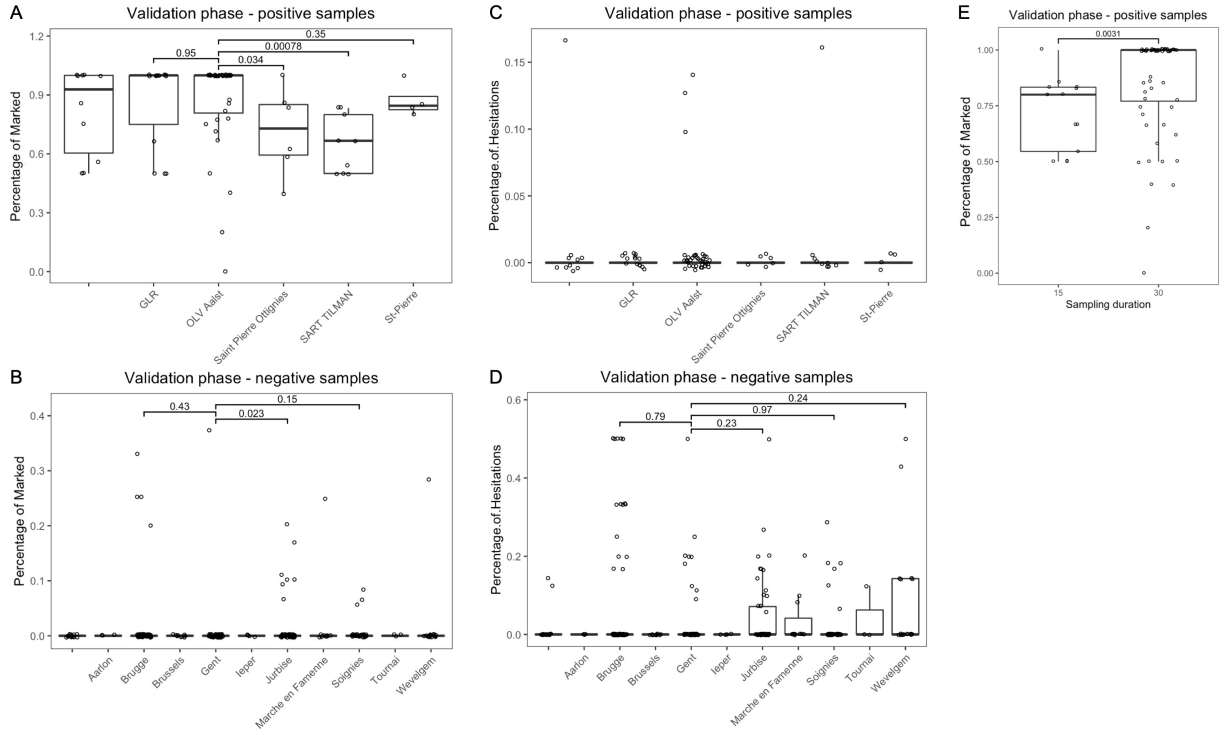

FIGURE 2
Impact of sampling location and sampling time on marking by the six trained SARS-CoV-2 detection dogs during validation phase. **(A)** Impact of sampling location on percentage of marking by the detection dogs in the SARS-CoV-2 positive samples (from hospitals). **(B)** Impact of sampling location on percentage of marking by the detection dogs in the SARS-CoV-2 negative samples (from volunteers). **(C)** Impact of sampling location on percentage of hesitations by the detection dogs in the SARS-CoV-2 positive samples (from hospitals). **(D)** Impact of sampling location on percentage of hesitations by the detection dogs in the SARS-CoV-2 negative samples (from volunteers). **(E)** Impact of sampling duration on percentage of marking by the detection dogs in the SARS-CoV-2 positive samples (from hospitals).

and found to have a significant correlation with the marking percentage by the detection dogs. Surprisingly, the number of markings on the negative samples was higher if volunteers reported to not have used medication. This is likely a confounding factor to the sampling location, as the full survey completion varied across locations.

GC/MS

This study aimed to identify the volatiles that are detected by the dogs and marked as positive. Our results indicate that SARS-CoV-2-positive samples indeed contained different signature volatiles that were significantly less present in SARS-CoV-2-negative sweat samples (Figure 3A). The detection dogs did not pick up one single compound, but rather a wide variety of different volatiles (Figure 3; Table 3; Supplementary Figure S4). Several classes of volatiles were repeatedly found as significantly enriched in SARS-CoV-2-positive as compared to SARS-CoV-2-negative samples. Five volatiles structurally related to 1-octan-3-ol were significantly associated with positive sweat samples, and were not found in SARS-CoV-2-negative samples (Figure 3B and Table 3). Seven volatiles which were structurally related to DL-3,4-dihydroxymandelic acid, and its metabolites, were similarly associated with positive sweat samples (Figure 3B and Table 3). Urocanic acid and its metabolites were detected several times and significantly linked to SARS-CoV-2-positive samples (Figure 3B and Table 3). Octadecyl acetate and its derivatives were another important group of volatiles detected in the positive samples (Figure 3B and Table 3). These and a series of other (unknown) volatiles form the unique scent that the detection dogs picked up and assigned as positive sweat samples.

Acceptability results of SARS-CoV-2 detection dogs

A large majority of the responders (76.2%) fully agree that dogs can be used to diagnose SARS-CoV-2 infection (Figure 4A). And an even larger majority (81.2%) of the responders fully agree that dogs can be used to diagnose SARS-CoV-2 infection based on a sweat sample (results not shown). The outcome of the corona dog outcome was similarly trusted by the responders (45.1%) (Figure 4B). About 34.8% would likely trust the outcome and 15.5% would maybe trust the outcome of the detection dog. However, there were still some doubts among the trustworthiness, mostly as the qPCR test result was trusted better ($p < 0.001$). Still, if we asked which test would be trusted more, the majority of the responders (45.3%) did not know which test would be the most trustworthy: the qPCR test result or the corona dog test result (Figure 4C).

The large majority (78.4%) of the responders did not have any ethical problems with the use of detection dogs to trace SARS-CoV-2 with people (Figure 4D). Nonetheless, 21.6% of the responders had some form of ethical questions around the use of dogs for this

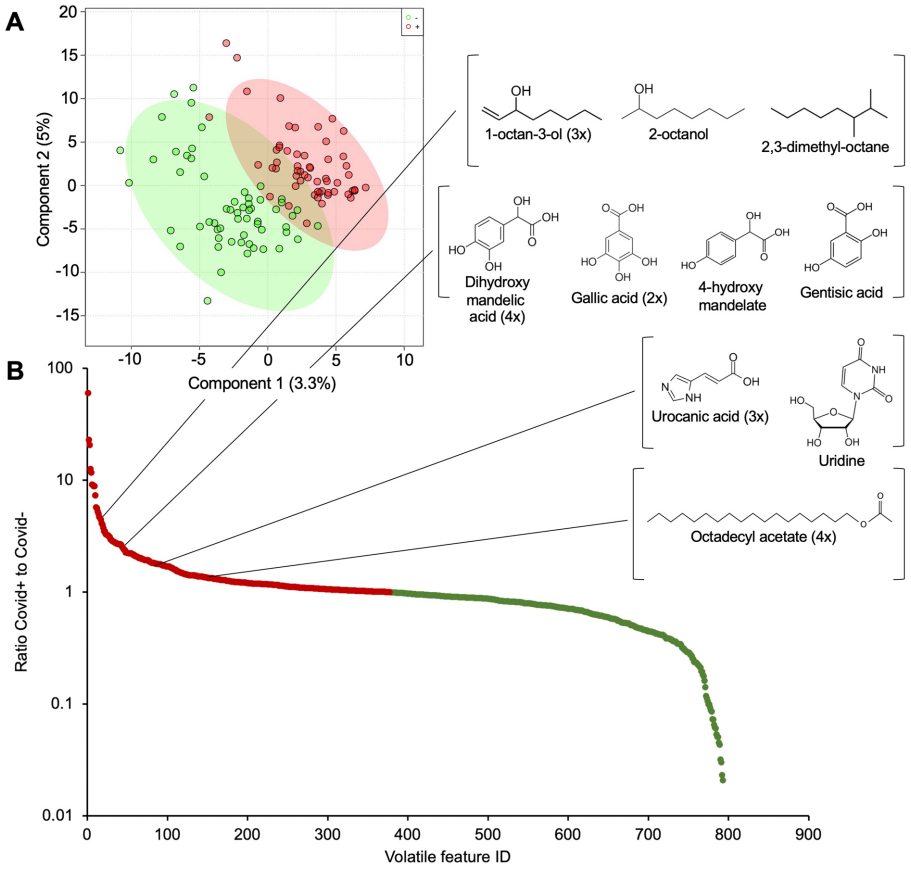

FIGURE 3
Molecular exploration of differences between SARS-CoV-2-positive and SARS-CoV-2-negative sweat samples. **(A)** PLSDA plot indicates clear differences in metabolic background among the SARS-CoV-2-positive (red) and SARS-CoV-2-negative (green) sweat samples. **(B)** The plot shows the ratio of abundances of volatiles in SARS-CoV-2-positive versus SARS-CoV-2-negative sweat samples. Ratios higher and lower than one (i.e., volatiles that are enriched in SARS-CoV-2+ versus SARS-CoV-2- samples) are highlighted in red and green, respectively. Examples of important and recurrent annotated volatiles and their respective molecular related volatiles are depicted.

purpose. The ethical concerns were the major confounding factor in the general acceptance of the corona dogs in practice ($p < 0.001$). It was also a main confounding factor in the general trust in the outcome of the SARS-CoV-2 detection dogs ($p < 0.001$) (Supplementary Figure S5A). This suggests that the ethical considerations surrounding the use of detection dogs played a pivotal role in shaping both the acceptance and trust as a detection method. The majority (69.3%) of the responders expressed no concerns in the practical organization of the SARS-CoV-2 detection dogs to sniff SARS-CoV-2 infection with people (Figure 4E). There were nonetheless 30.7% of the responders that did have some doubts on the logistical aspects of employing SARS-CoV-2 detection dogs. These practical doubts were also a main confounding factor in the general acceptance of the corona dogs in practice ($p < 0.001$) (Supplementary Figure S6A).

The large majority (74.8%) of the responders found it safe to use dogs for the purpose of detecting SARS-CoV-2 infection with people (Figure 4F). There were however still some doubts on the safety of using sniffer dogs and it was the second biggest confounding factor in the general acceptance of SARS-CoV-2 detection dogs in practice ($p < 0.001$) and the second biggest confounding factor in the general trust in the outcome of the SARS-CoV-2 detection dogs ($p < 0.001$) (Supplementary Figure S5C). Fear of dogs was a confounding factor in the general acceptability ($p < 0.001$) and in the general trust of the outcome of the detection dog ($p < 0.001$). A dog allergy or religious problems with dogs did not have any significant relationship with the general acceptability in the SARS-CoV-2 detection dogs ($p = 0.8385$ and $p = 0.6636$, respectively).

A large majority (77.8%) was willing to donate their armpit sweat for the purpose of training SARS-CoV-2 detection dogs. The willingness to share their armpit sweat to train detection dogs was also a confounding factor in the acceptance of the responders ($p < 0.001$). However, when asking which sample the responders would be most likely to give for the SARS-CoV-2 detection dog to evaluate, the majority indicated they would rather give a sample of their armpit sweat (64.2% of the responders) than a nasal sample (24.3% of the responders). Other samples that the responders are willing to provide are their mouth mask (70.0% of the responders) and a sample of their saliva (66.9% of the responders). However, when needing to take a decision on which test to take when arriving at the airport, most people preferred the dog test (60.6%) or the dog test together with a qPCR test (27.7%) over the qPCR test (4.6%) or another fast test (7.1%) (Supplementary Figure S6B). The responders indicate the

TABLE 3 SARS-CoV-2-positive associated volatiles, with *p*-value and putative feature identity.

Retention time	Covid	*p*-value	Feature ID
2.03	+	7.61E-06	1-octen-3-ol
2.46	+	0.0045	2-octanol
5.60	+	0.0013	1-octen-3-ol
5.60	+	0.0413	1-octen-3-ol
16.11	+	0.0386	2,3-dimethyl-octane
4.66	+	0.0094	Gallic acid
5.94	+	0.0002	Gallic acid
6.45	+	0.0103	4-Hydroxymandelate
6.57	+	0.0317	DL-3,4-Dihydroxymandelic acid
6.65	+	0.0418	Gentisic acid
6.95	+	3.24E-05	DL-3,4-Dihydroxymandelic acid
7.98	+	0.0007	DL-3,4-Dihydroxymandelic acid
9.28	+	0.0002	Urocanic acid
9.28	+	0.0227	Urocanic acid
9.82	+	0.0073	Urocanic acid
10.23	+	6.16E-05	Uridine
11.99	+	0.0465	Octadecyl acetate
12.11	+	0.0012	Octadecyl acetate
12.17	+	0.0251	Octadecyl acetate
12.50	+	0.0177	Octadecyl acetate
3.22	+	0.0360	2-methyl-N-ethyl-N-octadecyl-propanamide
3.32	+	0.0079	2-methyl-N-ethyl-N-octadecyl-propanamide
3.76	+	0.0004	Unknown
4.11	+	0.0454	Hexanal
4.53	+	0.0053	3-Acetoxy-2-chlorpromazine
7.01	+	0.0112	N-phenyl-benzenemethanamine
8.62	+	0.0208	Camphor
8.72	+	0.0113	Camphor
10.79	+	0.0251	trans-2-tert-butyl cyclohexanol acetate
11.33	+	0.0218	L-Ascorbic acid
12.69	+	0.0019	(6Z,9Z)-6,9-Hentriacontadiene
12.95	+	0.0248	(6Z,9Z)-6,9-Hentriacontadiene
13.28	+	0.0294	3,4-Dihydro-2,5,7,8-trimethyl-2-benzyloxycarbonyl-2H-1-benzopyran-6-ol
16.74	+	0.0335	N-hexyl-acrylamide
18.14	+	0.0205	18-Nonadecenoic acid
20.65	+	0.0341	Tetradacanal
21.87	+	0.0213	unknown

unpleasant feeling that is associated with taking a sample for a qPCR test and the convenience of providing a sample for the SARS-CoV-2 detection dog to evaluate.

Communication on the SARS-CoV-2 detection dogs played a significant role in influencing the general acceptability and trust in the SARS-CoV-2 detection dogs (Supplementary Figure S5B). Respondents who had prior exposure to information about these detection dogs, through press and social media, exhibited higher levels of acceptance ($p < 0.001$), with communication resulting in a 30% increase in acceptability for practical implementation. Communication also had a significant impact on the general trustworthiness of the SARS-CoV-2 detection dogs ($p < 0.001$). The age of the responders also played a significant influence on the overall acceptability and trust in the detection dogs (Supplementary Figure S5D). Younger age groups displayed lower levels of acceptance ($p < 0.001$) and general trustworthiness in the outcome of the detection dogs ($p < 0.001$), including concerns related to potential refusal at the border or entrance of an event based on the result of SARS-CoV-2 detection dogs ($p < 0.001$). When asked about the location where to deploy the corona dogs, the responders preferred to use them in the airport (88.4% of the responders), cultural events (78.0% of the responders), and sports events (70.9% of the responders) (Supplementary Figures S6C,D). The language group (2,261 Dutch speaking and 1,330 French speaking) did not have any influence in the general acceptability or trust in the SARS-CoV-2 detection dogs. The biological gender also did not have a significant correlation with the general acceptance on the use of SARS-CoV-2 detection dogs, nor in the general trust in the outcome of the detection dogs.

Discussion

The trained SARS-CoV-2 dogs demonstrated an overall accuracy of 95% after the validation phase. The average sensitivity, measuring the ability to correctly identify positive cases, was 81%, while the average specificity, indicating the ability to correctly identify negative cases, was 98%. The performance measures remained consistent during the post-validation stage, with an average sensitivity of 80% and average specificity of 99%. These results are in accordance with the general recommendations set by the European Center for Disease Prevention and Control and the World Health Organization, thereby requiring minimum 80% sensitivity and minimum 97% specificity for a valid SARS-CoV-2 test. Therefore, the SARS-CoV-2 detection dogs can be considered as reliable rapid antigen tests.

The results presented in the validation and post-validation phase are comparable with those of other studies. Other research groups have used different samples to train the detection dogs (19): 1/ armpit sweat (12–14, 20–22), or 2/ saliva or tracheobronchial secretion (13, 23, 24), or 3/ urine (13), or 4/ masks and clothes (24). Overall sensitivity and specificity, with all types of samples included, varied from 65 to 100%, and from 85 to 98%, respectively. While comparing studies working only with sweat samples, the sensitivity ranged from 71 to 100%, and the specificity ranged from 85 to 99%. We attempted to document the qPCR cycle threshold (Ct value) for samples included; however, this was not possible for each sample used throughout the study. We observed that the dogs could more easily detect samples from SARS-CoV-2 positive patients when Ct value was below 25.

The number of dogs in our training protocol was limited to 6, while we started with 13 at the beginning, as a number of dogs did not pass the initial selection tests. This corresponds to the range typically used in other studies, where the use of 6–12 dogs is common (13, 14,

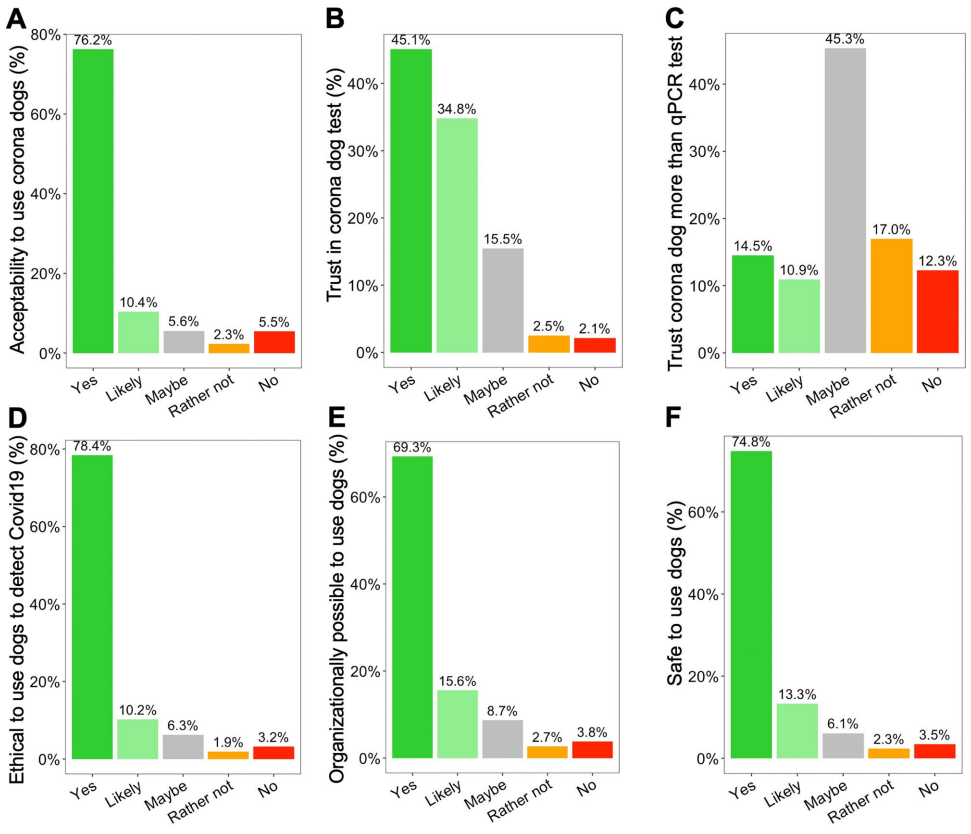

FIGURE 4
Results from the national survey on acceptability toward the use of SARS-CoV-2 detection dogs in practice. **(A)** Do you accept detection dogs to be used for this purpose? **(B)** Do you trust in the outcome of the SARS-CoV-2 detection dogs? **(C)** Which test would people trust more: the outcome of the SARS-CoV-2 detection dogs or the outcome of the qPCR test? **(D)** Is it ethical to use detection dogs for this purpose? **(E)** Is it organizationally possible to use detection dogs in real life? **(F)** Is it dangerous to use dogs for this purpose?

24–26). The performances of the 6 Belgian dogs exhibited similarity to that observed many other studies using armpit sweat samples.

During the training phase, the dogs performed 6 to 10 runs per day, 3 to 4 days per week, representing an intensive regimen designed to enhance their learning capacity. Careful consideration was given to prevent olfactory fatigue and performance decline by avoiding excessive runs that may induce exhaustion. In the validation phase, the dogs received training to 5 days per week, while after validation, the training frequency reduced to 1 day every 2 weeks. Consequently, the dogs were more relaxed, although the overall performances after validation were not significantly different compared to the validation phase.

Proper sampling methods are crucial for training effective detection dogs. It is essential to use samples that have been confirmed positive or negative through qPCR testing. Sampling time with the patients was a determining factor in successful identification by the detection dog. In our trial, samples from each patient/participant were transferred into plastic bags, frozen to −20°C, transported, stored for some time, thawed (30 min) (−20°C to 16°C) before being presented to the detection dog. However, this process can result in a certain loss of volatile molecules. To mitigate this loss, we aimed to ensure a minimum contact time between sample and armpit of 15 min, with the majority of samples having a contact time of 30 min. In contrast, other studies have generally employed shorter contact times (12–14, 20–22). Based on our experience, a longer initial sampling time, allowing for greater retention of odorous molecules in the cotton pads, is preferred to account for the losses during freezing, thawing, transportation and storage (27). In our study design, a sampling time of 30 min is preferred over 15 min. However, some studies have demonstrated good results with a contact time as short as 5 min (20), where samples were provided to the dogs fresh or stored at +4°C (21). In our study, all samples were stored at −20°C, as working with fresh samples risked evaporation of volatiles between sampling and training. Increasing the contact-time between cotton balls and armpit may improve the quality of the sample when freezing is involved, but it may not be practical in field conditions. The possible solution is to use fresh samples for testing, which would reduce the contact time between cotton balls and patients while maintaining good performances of the test.

During the validation phase, a rigorous approach was employed to ensure the reliability of the results. Unlike other studies, no empty cones devoid of human scent (blank) were used, but sweat samples worn by confirmed SARS-CoV-2 positive or negative cases were exclusively employed. Additionally, the dogs were exposed to completely negative series, consisting of samples coming from different volunteers. This rigorous method was not found in previous studies and was superior to other studies conducted on sweat samples. The validation process was undertaken in double-blinded conditions.

To prevent any sampling center-related bias, sweat samples were obtained from different locations, from patients with different clinical signs, and at different moments of the day, irrespective of the Ct-value. This comprehensive approach aimed to ensure the robustness of the results by avoiding any potential biases associated with the sampling center.

At the beginning of the training, cotton balls and gauze were used as scent carriers. In order to enable the dogs to discriminate the specific SARS-CoV-2 odor, it was essential to consistently present the same scent carriers for positive or negative samples. Almost all negative samples were collected on cotton balls so it was necessary to collect the positive samples on cotton balls as well. Otherwise, the dogs could learn to distinguish cotton from gauze, which is not wanted.

Variations in the performance of the dogs may come from a range of different factors, including their individual skills, specific function (explosive detection versus search and rescue), conditioning, training, age, diet and environment (28). In our study, we observed a potential lower performance when the outside temperature was higher than 25°C, despite conducting training sessions in a controlled environment with regulated temperature and humidity. The elevated temperatures could exhaust the dogs in between the runs. It is known that dehydration of the mucosal layer in the canine nasal cavity can significantly decrease odor detection capabilities (29, 30).

During the training phase, the dogs had the tendency to show hesitation at both the first and last cones. In future training setups, it would be preferable to arrange the cones in a circular way to address this issue. In order to improve the sensitivity of the dogs, double validation (2 dogs testing the same sweat samples) could be performed on real samples. Previous studies have reported a sensitivity of up to 100% using this double validation (22). The specificity of the 6 trained dogs was overall very high. This is an interesting characteristic because it allows SARS-CoV-2 negative people to not be stuck in an airport or other place/event in case of a false positive result. Despite a high specificity, confirmed negative patients did not have another qPCR test several days later to confirm the result.

There was no significant correlation found between the age, biological gender, body mass index, or deodorant use of the participant that provided the sample and the detection (or hesitation) by the trained dogs. This is good news, as this means that deodorants cannot cover up an underlying SARS-CoV-2 infection of a patient. Similarly, individual characteristics such as biological gender, age and body mass index do not influence the efficacy for trained dogs to detect an underlying infection. This makes the detection dog method a robust and uniform detection method for SARS-CoV-2.

The trained SARS-CoV-2 dogs correctly identified 100% of vaccinated people as negative, and thus healthy. The individuals received the Comirnaty® vaccine in two doses and were sampled 3 weeks after the second dose. At least after 3 weeks, the vaccination process did not interfere with the dogs' ability to distinguish positive and negative samples, thus avoiding false positive detection in healthy vaccinated people.

The trained detection dogs were able to detect a mixture of different volatiles (Figure 3; Table 3; Supplementary Figure S4). Particular volatiles were repeatedly retrieved and significantly associated with SARS-CoV-2 positive sweat samples, and not associated with SARS-CoV-2 negative sweat samples. Some of the volatiles were breakdown compounds and could be traced back. 1-octen-3-ol, detected in higher abundances in SARS-CoV-2-positive sweat samples, is known to be secreted by human skin and is an important attractant for mosquitoes (31). It is a breakdown product of linoleic acid, which was identified as an important antiviral fatty acid. A study showed that linoleic acid was the most antiviral against the SARS-CoV-2 virus, with a direct binding to the cavity formed by the RNA double helix and protein (32). As such, it is hypothesized that 1-octen-3-ol is present in sweat in higher amounts because of the activity of linoleic acid. These alcohols have been found in the breath of (critically ill) SARS-CoV-2 patients (19, 33–35) which confirms their elevation with SARS-CoV-2 infection. DL-3,4-dihydroxymandelic acid and its derivatives are metabolites of norepinephrine and have antioxidant properties (36). Norepinephrine is an important hormone and neurotransmitter in the human body which is released in higher levels during situations of stress or danger (37). Norepinephrine is also a known neurotransmitter in the Merkel cells located in the skin (38). Norepinephrine (or a structurally related neurotransmitter) may be implied in the cytokine storms present in SARS-CoV-2 patients (39, 40). In that case, the dogs can detect the neurotransmitter metabolites that are implied in the cytokine storms of SARS-CoV-2 patients. Several volatiles related to urocanic acid were detected in higher abundances in SARS-CoV-2-positive samples. Urocanic acid is naturally present in human sweat and in the stratum corneum and is a breakdown product of filaggrin (41). It is known to act as a photo protectant and absorbs UVB light (41). It could be upregulated in SARS-CoV-2-positive sweat samples as part of the natural immune reaction of the human body to the Sars-CoV-2 virus, although the real reason remains to be elucidated. Octadecyl acetate was similarly repeatedly found and associated with SARS-CoV-2 positive sweat samples. It remains unclear why these volatiles are upregulated. A variety of other volatiles have been identified (Table 3) and all add up to the unique scent associated with infection that the detection dogs were able to pick up.

Detection dogs are widely used to detect narcotics and explosives (42), however, to the public, it is relatively unknown that detection dogs can be used to detect SARS-CoV-2 infection in humans. The Belgian population largely supports the SARS-CoV-2 detection dogs as a valid detection technique however; a minority of people raised some constraints (Supplementary Figure S5). The use of a quick antigen test seems to be easier to handle compared to detection dogs (43). Some respondents were afraid of dogs but this can be solved by avoiding direct contact between a screened person and a detection dog. Communication on the possibilities of SARS-CoV-2 detection dogs was very important in order to increase overall acceptance as a valid SARS-CoV-2 detection test. Nonetheless, based on our large Belgian survey, most of the Belgian people were open toward the use of detection dogs to detect SARS-CoV-2.

In general, people were more open toward detection by a dog, rather than taking a nasal sample for qPCR test. The latter is more invasive as compared to taking a sweat sample (44). Respondents also preferred to provide an armpit sweat sample, rather than socks, urine, shirt, or neck sweat sample. Providing an armpit sweat sample is one of the least invasive methods to detect on SARS-CoV-2, together with providing a disposable mouth mask and saliva. The Belgian respondents saw great value in using the detection dogs at the airport. Other preferred locations were cultural and sports events. Detection dogs would indeed be very useful in these locations, where large numbers of people can be screened in a small amount of time and with

minimal efforts (45). The main reservations that people had toward such detection dogs were related to ethical considerations, safety and organizational considerations. Similar ethical considerations have been noted before (19).

The current study has limitations. Ideally, the dogs can also detect asymptomatic people, but we had only few of these samples during training. The main objective was to train detection dogs on the ability to distinguish between SARS-CoV-2 infected and healthy people, and we therefore primarily trained with positive samples coming from patients with clear symptoms and qPCR results of <25 cycles.

We did not confirm the absence of SARS-CoV-2 virus in the dogs' nose after training as the probability was very low. There was no direct skin contact between the sample and the dog's nose. It is also well known that sweat is not a classical way of virus excretion. Finally, the virus replicates very rarely in dogs (46), and only after prolonged and direct contact with a highly contagious patient. Therefore, the chance of viral transmission is minimal. Dog trainers were tested regularly and were not tested as positive. In addition, no dog or trainer got ill during the training, validation or post-validation.

The Belgian government supported the training of our SARS-CoV-2 detection dogs and acknowledged the great results obtained. However, the government wished to deploy the dogs directly among a crowd of people, which required a more specific training. Since the vaccines and the quick antigen tests were widely available, the Belgian government did not further support the detection dogs' program.

Conclusion

Detection dogs can be efficiently trained to detect SARS-CoV-2 based on sweat samples obtained from the armpit. The trained dogs exhibit a high specificity, rarely indicating a negative sample as positive. Ensuring the collection of high-quality samples is crucial, involving a consistent sampling protocol, with the use of the same carrier and sufficient odor captured. An appropriate storage at cold temperature (4°C or −18°C or −20°C) and 30 min sampling time is preferred, accompanied by confirmation of the sample being positive or negative through qPCR testing. Positive samples from symptomatic patients and samples sourced from different hospitals are recommended to avoid center bias.

A training protocol was constructed whereby the goal was to have 6 to 10 runs per day per dog. This range was determined to be sufficient for effective training, as fewer runs were insufficient to train the dogs, while more runs resulted in dog exhaustion. Additionally, detection dogs which were pre-trained to detect 'in line' were found to be easier for the training on SARS-CoV-2 samples. The dogs detected a wide variety of volatiles, among which a series of breakdown compounds of antiviral fatty acids and neurotransmitters/hormones.

The general public demonstrated a high acceptability toward the utilization of canines as SARS-CoV-2 detection tools. However, it is crucial to establish a proper practical setup. Direct contact between screened person and detection dog should be avoided, to deal with the fear that some people have. Enhancing overall acceptability requires effective communication regarding the possibilities and efficacy of the SARS-CoV-2 detection dogs. Sampling of the armpit for this purpose is preferred over a nasal swab for qPCR test, as it offers a less invasive approach.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving animals were reviewed and approved by the University of Liege (approval number N°20-2246). The studies involving human participants were reviewed and approved by Ethical Committee UZ Gent, approval number multicentric study BC-08571, as well as all participating hospitals (listed under Materials & Methods). The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any identifiable images or data included in this article.

Author contributions

CC, MS, FG, and HG designed the experiments. MP, RV, BaM, EV, PV, CC, and HG did the metadata collection. MP, RV, BaM, EV, and PVG trained the detection dogs and did the experimental organization and setup. AP, BeM, GD, SP, LDV, FS, KVV, SoT, AO, IM, PV, SJ, LV, ET, GW, J-CM, KA, LD'H, SeT, BDT, and JC collected samples from patients. AL, AM, and AA did the GC/MS analysis and putative identification of volatiles. DG provided the initial dog training and supplied feedback. CC and HG wrote the manuscript. CC made the figures and did the statistical analysis. All authors critically revised the manuscript for important intellectual content.

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Conflict of interest

CC is the founder of Armpit BV. AA and AM are founders of Arome Science Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer A-LC is currently co-organizing a Research Topic with one of the authors DG.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2023.1185779/full#supplementary-material

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