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Canine scent detection of sinonasal-inverted papilloma in blood plasma and nasal secretions

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ABSTRACT

Sinonasal-inverted papilloma (SNIP) is a rare, benign tumor of the sinonasal tract. Although these tumors are inherently benign, they are prone to malignant transformation and are associated with an unusually high rate of recurrence, even after complete surgical resection. As such, these patients must be followed long-term to monitor for recurrence or the presence of cancer. Unfortunately, there are currently no standards for long-term surveillance, and protocols usually require routine radiography and/or nasal endoscopy, which are not highly sensitive or specific, are time consuming, expensive, and confer morbidity to the patient. There is evidence that suggests that SNIP is associated with a uniquely detectable volatile metabolite signature. This study investigates the ability of four privately owned dogs to learn to identify the presence of SNIP in patients based on blood plasma samples. This type of medical detection is currently being used to create early diagnostic tools for other disease processes, which suggests promising results for the outcome of this study. Blood/plasma and nasal secretions were collected from both patients with SNIP, and healthy age-, sex-, and smoking-status-matched controls. Training was conducted twice weekly using errorless learning techniques that allowed the dogs to utilize odor concentration to guide their understanding of the target odor. After further training, a subsequent double-blind testing phase was conducted, in which the dogs' ability to expand recognition of the target odor from blood plasma samples to nasal secretion samples was assessed. Analysis of recorded training sessions revealed that the dogs exhibited successful odor discrimination between SNIP-positive plasma samples and SNIP-negative controls. Their pattern of behavior change also suggested similarity between SNIP odor in plasma and nasal secretions.
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Introduction

Sinonasal-inverted papilloma (SNIP) is a benign but locally destructive tumor of the nasal cavity and paranasal sinuses. SNIP is unlike other benign sinonasal tumors that have a potential risk of malignant transformation and a high rate of recurrence. Despite current surgical techniques, recurrence rates remain between 3% and 17%, and some studies suggest that these recurrence rates might be even higher (Zydroń et al., 2016; Bugter et al., 2017). As such, a technique to allow for early detection of the disease and more importantly, disease recurrence, is critical. Current standards require biopsies of the suspicious tumor to make the diagnosis. However, one study showed that 17% of biopsies from SNIP patients were incorrectly diagnosed as nasal polyps (Klimek et al., 2000). In addition, there are no accepted standards for surveillance, which often include routine imaging studies and nasal endoscopy. However, these techniques are neither highly sensitive nor specific (Lai et al., 1999; Suh and Chiu, 2014), and cases of subtle recurrence may be missed.

Abbreviations: SNIP, sinonasal-inverted papilloma; GC–MS, gas chromatography–mass spectrometry; VOC, volatile organic compound; PVWDC, Penn Vet Working Dog Center; UDC, universal detection calibrant; LDA, linear discriminant analysis

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Finally, patient attrition in monitoring programs further complicates long-term surveillance of the disease (Bugter et al., 2017). Given these difficulties with current monitoring methods, a minimally invasive diagnostic method that does not require precise biopsy, with high sensitivity and specify, could lead to lower rates of attrition and more accurate and timely diagnoses.

One potential avenue for SNIP detection and monitoring is to identify and utilize a signature odor profile for diagnosis. One recent study used gas chromatography–mass spectrometry (GC–MS) to identify volatile metabolites (also known as volatile organic compounds, or VOCs) in blood plasma and nasal secretions from patients with SNIP and demonstrated that the signature of these compounds could be used in a model to discriminate between SNIP patients and healthy controls (Chaskes et al., 2022). Chromatography was used to identify specific compounds in the headspaces (the gaseous space above the liquid specimen) of a plasma sample mixture and nasal sample mixture from SNIP-positive patients. Interestingly, while toluene was singularly predictive of SNIP in blood plasma samples, it was not singularly predictive in nasal secretions (Chaskes et al., 2022). The number of identified predictive VOCs in SNIP blood plasma and nasal secretions was different. As such, a universal SNIP VOC signature for both blood plasma and nasal secretions has not been identified.

Trained odor detection canines can also be used to identify odor signatures of disease (Cornu et al., 2011; Essler et al., 2021b; Horvath et al., 2013; Jendrny et al., 2021; Kane et al., 2022). Canine olfaction is incredibly sensitive and allows dogs to detect odors as low as one part per trillion (Ashton et al., 1957; Johnston, 1999). Dogs are often used by the military and police to find explosives (Gazit and Terkel, 2003; Lazarowski and Dorman, 2014), narcotics (Rice and Velasco, 2021), people (Bulanda, 2012), as well as many other targets. Dogs identify these targets by utilizing the VOC signatures that emanate from the sources (Angle et al., 2016). Dogs can also detect many different types of infectious diseases (Taylor et al., 2018; Essler et al., 2021b; Chaber et al., 2022), and metabolic-state characteristics of different medical conditions, such as hypo- and hyperglycemia (Hardin et al., 2015; Rooney et al., 2019) as well as seizures (Davis, 2017). Trained detection dogs have been shown to detect various types of cancers, including ovarian cancer (Horvath et al., 2013; Murarka et al., 2019), lung cancer (Buszewski et al., 2012), prostate cancer (Cornu et al., 2011), and others (Willis et al., 2004; Moser and McCulloch, 2010; Sonoda et al., 2011). These diseases and cancers have been detected in several different mediums, including blood plasma (Horvath et al., 2013; Rooney et al., 2019), urine (Cornu et al., 2011; Essler et al., 2021b), and sweat (Grandjean et al., 2020). As such, detection dogs could aid in identification of a universal SNIP VOC signature, and to corroborate the results produced by the GC–MS.

The first goal of this study was to determine whether trained detection dogs, similar to the GC–MS analysis and subsequent model created by Chaskes et al. (2022), could detect a unique odor signature of SNIP samples and discriminate this odor from healthy controls. The second goal of this study was to use dogs' behavior change patterns at odor to examine the similarity of the SNIP blood plasma sample odor to SNIP nasal secretion, a medium on which dogs have not been trained and had not been shown before this study.

Methods

Participants

Following Institutional Animal Care and Use Approval (protocol #806722) and informed client consent, four privately owned dogs were enrolled in this study. All dogs were trained in scent detection using an odor wheel by a Penn Vet Working Dog Center (PVWDC) trainer. Dogs varied in breed and sex and ranged in age from 2 to 8 years old. One of the oldest dogs (Pacy) was previously trained by the PVWDC, currently works in human remains detection, and has participated in previous odor studies at the PVWDC (Essler et al., 2021a). The youngest dog (Xoxo) had prior scent detection training. The two other participants (Crunch, Kodi) were recruited for this study along with their handlers after at least 6 months of scent training and a minimum stand-and-stare alert duration of 2 seconds on the PVWDC scent wheel (Essler et al., 2020).

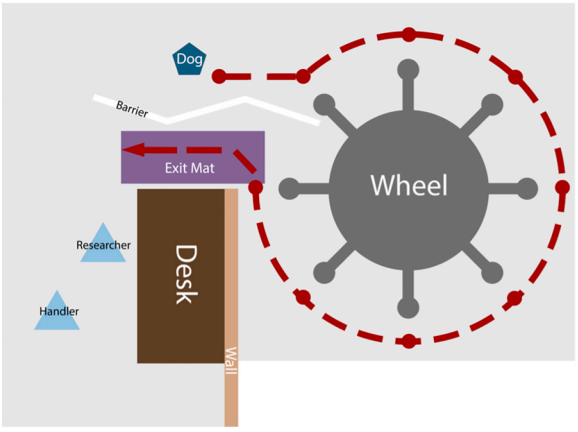

Figure 1. A diagram of the orientation of the scent wheel, dog, and handler within the study room.

Mechanics

Before this study, all dogs were trained on universal detection calibrant (UDC), a synthetic odor that allowed dogs to learn the mechanics of the odor wheel before learning the odor of interest (Furton and Beltz, 2017). The dogs were taught to search an eight-port circular scent wheel (Figure 1), sniffing each of the ports in order without skipping a port, and stopping when they reached the target odor. Each dog was required to exhibit a distinct change in behavior to indicate the location of odor: Crunch, Kodi, and Xoxo exhibited a “stand-and-stare-at-port” indication for a minimum of 2 seconds, while Pacy exhibited a sit-at-port alert. All dogs were trained to demonstrate a blank wheel behavior in which their back was to the odor wheel to signal to their handler that they did not recognize the target odor in any of the ports presented to them. The blank behaviors were all performed on the exit mat labeled in Figure 1, and ranged from a sit, a down, or standing still. After performing a correct alert, dogs were positively reinforced by their handler. Correct behavior was marked with a conditioned secondary reinforcer, an audible click, from their handler, followed by delivery of their preferred method of reward (either food or toy) after leaving the wheel. If the dog exhibited an incorrect response, they were allowed to finish searching the wheel before being called out of the wheel with no reinforcement and were immediately sent to search for odor again. If the dog searched all eight ports and incorrectly indicated a blank wheel, they were sent back into the wheel without reinforcement and allowed to attempt the trial again. If the dog correctly alerted to odor during any trial following their false-negative alert, they were reinforced for this alert during training, but the researchers considered this a failed trial. If any handler believed that it would be more beneficial for the dog to move on to the next trial without any further attempts on the wheel, that trial was skipped, and it was indicated in the data that the dog never exhibited a correct alert. The training mechanics utilized for UDC detection were identical to those necessary for this study.

Table 1
Dog information.

Dog name	Age (years)	Sex	Years of scent detection experience	Breed	Previous odors	Preferred reward
Pacy	8	FS	8	Labrador retriever	Human remains, UDC	Food: kibble
Crunch	5	MC	1	Pembroke Welsh corgi	UDC	Food: kibble
Kodi	8	FS	2	Labrador retriever	UDC	Food: hot dogs, meatballs, or apple slices
Xoxo	2	F	2	Belgian Malinois	UDC, live find (human odor)	Toy: tugging with handler

FS = female spayed, MC = male castrated, f =female, UDC = universal detection calibrant.

The test sessions that followed the completion of each dog's SNIP training were run double-blind, meaning the handler had no indication of where the positive sample was in the wheel and the researchers exited the testing area before the start of each test trial. Because the handler had no way to confirm whether a dog's final alert in a test trial was accurate, the handlers were asked to refrain from giving the dogs any feedback during testing, even if a final alert was offered. Dogs in test trials were asked to smell all eight ports regardless of whether they exhibited a final alert behavior; if the dog exited the wheel before smelling all ports, they were sent back to the wheel as they would be at the beginning of a new trial. Once dogs completed a full search of the wheel, they were given a mild verbal reinforcement (as opposed to the full click-and-reward reinforcement delivered during training trials) and brought fully out of sight of the wheel by their handler. The handlers then asked their dog to execute a simple obedience command (such as a sit, down, touch, or heel) meant to be completely removed from the tasks asked of them in the context of the wheel, while the positions of ports were switched by observers for the next trial. After performing the obedience command, the dog's behavior was marked with a click followed by their preferred reward (Table 1).

The trials in which the dogs received no reinforcement (referred to as “no-click trials”) were introduced to dogs before the testing phase. Exposing dogs to these “mock test” trials during their training gradually reduced frustration behaviors exhibited because of the lack of reinforcement for final alert behaviors on target odor.

Specimen collection and processing

Twenty-four patients over the age of 18 with SNIP were recruited and consented to participate. Approximately 20 cc of whole blood was collected from each patient. This sample was centrifuged for 20 minutes at room temperature at a rate of 2000 rpm. The plasma was then transferred to a glass tube and stored at − 80 °C. In addition, nasal secretions were collected from each patient in a Lukens trap before the administration of any intranasal topical decongestant. The nasal secretions were transferred directly to a glass tube, without additional processing, and stored at − 80 °C. All specimen samples were stored in an onsite biorepository. Whole blood, plasma, and nasal secretions were always stored in glass vials to mitigate the risk of potential contamination from plastic containers. All specimens were collected immediately after the induction of anesthesia, before the initiation of surgery, to mitigate the risk of potential surgical confounders.

Twenty-seven healthy controls over the age of 18 undergoing nasal surgery without sinonasal pathology (i.e., rhinoplasty, endoscopic dacryocystorhinostomy) were recruited and consented in a similar fashion. Controls were age, sex, and smoking status matched (Table 1). Again, whole blood and nasal secretions were collected and stored as described above (Table 2).

Fourteen control blood plasma samples and 21 SNIP-positive blood plasma samples were used during training. In addition to the negative controls (provided by one of the authors, M.R.R.), 54 healthy control blood plasma samples from a prior ovarian cancer detection study were utilized throughout training and testing as controls (Kane et al., 2022). It should be noted that these samples were not tested for SNIP; however, they came from asymptomatic healthy patients. All negative testing samples came from the set provided by one of the authors (M.R.R.) and were confirmed negative for SNIP.

Table 2
Demographic and clinical data by diagnosis.

	SNIP	Control	*P*-value
Age (average in years)	56.4 ± 15.0	54.8 ± 16.1	0.246
Gender			0.435
Male	13	14	
Female	11	13	
Smoking status			0.707
Current	11	14	
Former	10	10	
Never	3	3	

Dogs were trained on the target odor from blood plasma samples and were tested on odor recognition utilizing blood plasma samples followed by novel nasal secretion samples. Fifty (50) microliters of either blood plasma or nasal secretions were pipetted from aliquots provided to the PVWDC by senior clinical authors (M.R., G.N., E.T., and M.R.) and deposited into small jars (about 4.5 cm in diameter, 5 cm in height) with metal lids. Sample jars were labeled with the patient ID, the appropriate positive or negative status indicated by a plus sign (+) or minus sign (−), and the date on which aliquots were sampled. All aliquots and samples were stored in a − 80 °C freezer between uses. Samples were thawed at room temperature for approximately 5 minutes before being presented to the dogs.

In total, 21 positive samples and 68 negative samples were available for training. Three (3) of the positive samples and six (6) of the negative samples were not used for training and were reserved for the test phase.

Errorless learning

In the training phase, the dogs were trained to alert on blood plasma from patients with SNIP and differentiate these samples from those from healthy individuals and distractors such as alcohol, cotton balls, or nitrile gloves. The position and presence of each sample in the wheel were randomized in every trial. As such, distractors, novel samples, and repeat samples are not spaced out in any particular pattern. Generally, every sample was moved to a different port or replaced with another sample between each trial. However, there were guidelines set in place to prevent excessive repeating of samples, and to ensure dogs were not using prior trials to inform their decision-making in the trials following. After samples were organized randomly throughout a session, researchers reviewed the sheet to ensure the following:

1. Samples, regardless of the status, were not placed in the same port more than two times in a row.
2. SNIP-negative samples were not placed in a port that previously contained a SNIP-positive sample, and vice versa.
3. SNIP-negative samples were not placed in the same port more than three times in succession.

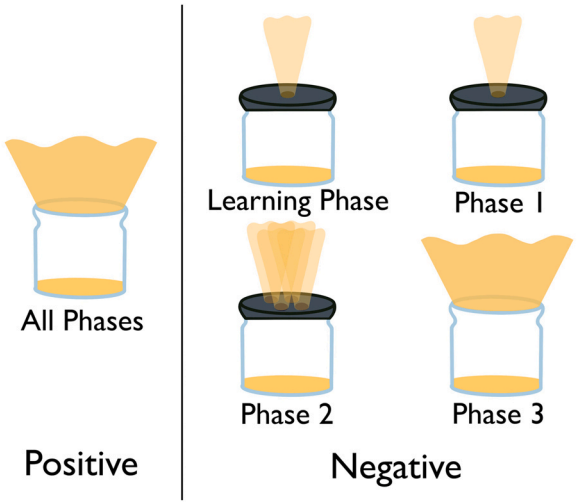

Figure 2. Visualization of how odor concentration was physically restricted throughout training.

These guidelines were followed during all phases of training and testing.

Dogs were introduced to the target odor using errorless learning techniques (Mueller et al., 2007; Essler et al., 2021b). Errorless learning involves providing the dog with a concentration cue as well as an odor cue, which in theory should make the identity of the target odor clearer for the dog. Gradually, the concentration cue was removed, and the dogs are asked to identify the target odor against controls when all samples are presented at the same concentration. The difference in concentration in this study was achieved by creating lids for sample jars with a varying number of holes punched through them to allow odor to escape (Figure 2). Lids were created with one and four holes, respectively (each hole measuring about 5 mm in diameter), to be presented to dogs during the different stages of training. The last stage of training involved the removal of the concentration cue; so both the positive and negative samples were presented in fully open sample jars. The final progression in an errorless learning training design requires the dog to recall the information obtained when the concentration cues are present in combination with the perceived differences in odor composition to differentiate between the target and control odors.

Initial odor learning

In the first phase of training, dogs were introduced to the odor via a single positive and negative sample, each placed within a separate port held by a researcher to eliminate providing any visual cues. The ports were held outside of the wheel by an experimenter wearing nitrile gloves, and the dog was directed to smell each port while their handler remained fully visible. The researcher was asked not to hold eye contact with the dogs and to refrain from making any noise while the dog was searching to allow the handler to be the most engaging person in the room. The positive sample was left completely open without a lid, while the negative sample was capped with a one-hole lid to limit the release of odor. All dogs were marked with a click and positively reinforced with a reward upon sniffing the positive SNIP sample and ignored while sniffing the negative sample. This process was repeated 10 times outside of the wheel.

The dogs then completed 10 trials on the wheel. The same positive and negative sample from the outside-wheel odor learning was used in the wheel. Four to six trials were *target* trials, containing a SNIP-positive odor and a SNIP-negative odor. The remaining trials were *blank* trials, containing no positive SNIP sample; however, the SNIP-negative sample was present in these blank trials. In both target and blank trials, the ports not containing SNIP-positive or SNIP-negative samples contained a subset of distractors that co-occurred with the preparation and handling of the samples (paper towel, nitrile glove, vinyl glove, alcohol, tape, sticky note, cotton ball, or pen cap). In this phase, seven distractors were used during blank trials alongside the SNIP-negative sample. Six distractors were used during target trials alongside the SNIP-negative and SNIP-positive samples. Distractors were randomly selected from the set of eight total distractors at the start of each session.

For each trial, the dogs were asked to search the wheel for the target odor, using the same mechanics taught during UDC training. The positions of all samples were changed by a researcher between each trial.

For this learning phase only, dogs were advanced to the next stage based on their trial accuracy rather than their initial reaction to odor. Initial reaction to odor is not possible in this learning phase because dogs are exposed to the odor outside the wheel before independently searching for the target odor in the wheel.

A target trial was considered to be correct if a dog passed the SNIP-negative sample and alerted on the SNIP-positive sample. A blank trial was considered to be correct if the dog passed the SNIP-negative sample and performed their blank behavior after searching all the odors. If dogs correctly responded to 9 out of 10 trials in this phase, dogs were progressed to Phase 1. The only difference between Phase 1 and the learning phase is that in Phase 1, dogs were not shown the samples outside the wheel.

Phase 1

Dogs in Phase 1 were trained with open positive samples and one-hole-lid negative samples, just as in the learning phase. There were 10 trials in Phase 1, with 4-6 of those 10 trials consisting of blank trials.

In addition to the guidelines discussed in the previous section, Phases 1-3 were also reviewed following randomization to ensure that all positive samples (2) and all negative samples (4) appear at least once within a session. Every session contained 8-10 repeats of each control sample, 6-10 repeats of each selected negative sample, and 2-3 repeats of each selected positive sample.

While the odor concentration of the SNIP-negative sample remained the same as the learning phase, the odor *variation* increased, and the dogs were not presented with the odors before independently searching the wheel. In each session, dogs were presented with a total of four negative and two positive samples. In each target trial, dogs were presented with a maximum of one positive sample and three negative samples. In each blank trial, dogs were exposed to a maximum of four negative samples. The order and placement of these samples were randomly generated.

These negative and positive samples were each presented to dogs two to three times within a session depending on the number of blank trials (between 4 and 6). All samples were initially novel to all dogs apart from one negative sample they had been shown during the learning phase.

Each of the 10 trials within a session included at least two negative samples and contained the same distractors that were used upon the dogs' first introduction to odor. This was the first phase where dogs were asked to discriminate the target odor amidst biological variation present throughout varying positive samples. To move on to Phase 2, dogs needed to correctly alert on at least five novel positive samples and correctly ignore seven novel negative samples over three consecutive sessions. One dog, Crunch, did not hit benchmark, but was moved on to Phase 2 to preserve a set number of novel samples to be presented to him with a lesser concentration cue.

Phase 2

The concentration cue was lessened in Phase 2, so the one-hole lids used on negative samples were replaced with four-hole lids. This approach acted as an intermediate step between the introductory period of Phase 1 and the complete dependence on an odor cue necessary for Phase 3. The procedure in this phase was very similar to Phase 1, with the major difference being in the ratio of novel to repeated samples. Dogs were exposed to positive samples that were previously presented in the learning stage rather than completely novel samples to optimize use of the limited inventory. Samples were repeated such that dogs were only presented with a non-novel sample after at least 3 weeks had passed from the time it was last presented to them.

The benchmark for moving onto Phase 3 was the same as was required to move to Phase 2 of training. Two dogs, Crunch and Xoxo, did not reach the benchmark for positive samples, but were capped at six sessions and progressed onto the next phase. The six-session maximum was to prevent overtraining on non-novel samples while the concentration cue was still present (Essler et al., 2021b).

Mechanical errors were also identified in their training, which was independent of their odor recognition abilities (i.e., skipping ports, favoring ports, et cetera) but nonetheless affected their scores, subsequently hindering them from progressing through the study.

Phase 3

Phase 3 was the final stage of training in which dogs were exposed to all odors at full concentrations, and as such, no sample lids were necessary. The sample variation of this phase was analogous to the previous two phases, but the ratio of novel and non-novel samples was once again neutered. During the first six sessions of this phase, dogs were presented with two non-novel positives, three novel negatives, and one non-novel negative during each session. However, this ratio was changed to 2:2 for the following three sessions, and eventually dogs were only presented with one novel negative among three non-novel samples. Because the ratio of familiar to novel negatives shifted throughout Phase 3, sensitivity was calculated based on dogs' initial reactions to odor within each session. Because repetition was so frequent, researchers attempted to maximize time between the use of samples and randomized which samples were used in conjunction with one another in each session.

This phase also included the introduction of “no-click” trials (intermittent reward) intended to create a more gradual transition for dogs from training procedure to testing procedure. One “no-click” trial was inserted into each training session and was carried out as a blind test trial would be. The no-click trial was immediately followed by a normal test trial where only the position of the positive sample and a randomly selected distractor was switched. The rest of the samples remained in the same positions to minimize the time spent between the “no-click” and the traditional training trials. After the dogs completed the trial that followed the “no-click” trial, they were given an enthusiastic reward and their normal click-and-reward reinforcement.

Training in this phase was not limited by a maximum because of how similar the nature of the sessions was to the design of the testing phase; thus, a demonstration of significant recognition of odor (80% specificity and sensitivity over three sessions) was crucial before a dog was placed in a testing scenario.

Testing

Test sessions were designed as a progressive analysis of the dogs' comprehension of the target odor. The first test session assessed how well the dogs could identify the target odor among full concentration controls, as was presented to them in Phase 3. In test sessions two and three, dogs were asked to discriminate the target odor from a novel negative control when both samples were presented as nasal secretions.

Each test session contained only seven trials instead of 10, to minimize stress and potential frustration for the dogs. In each phase, dogs were asked to complete four training trials in a very similar manner to how they would complete a training session, as well as three blinded test trials. All sessions contained non-novel distractor controls and at least one non-novel negative sample.

The test trials contained one novel positive and one novel negative sample in each trial, as well as 4-5 distractor controls and 1-2 non-novel negative samples. Both novel samples were samples of the same medium, and all non-novel negative samples were blood plasma samples. The position of all ports was randomized before the study and the handlers remained completely blind to the sample placement in each test trial. Once the test trial samples were placed in the wheel by the researchers, they exited the room. The blinded handler then ran their dog on the wheel. This double-blind setup was used so that no one who knew the location of the target sample was present in the room while the dog was testing.

All training sessions utilized non-novel positives and negatives presented to the dogs in a randomized order just as they would be during a Phase 3 training session. When dogs were sent into the wheel to complete a training trial, their handlers delivered positive reinforcement after a final alert at a port containing a positive plasma sample or a correct blank alert. The first trial of each test session was designated as a training trial, and there were never more than two training trials in a row.

Final analyses were done using only novel positive and negative samples, to ensure that prior learning effects do not influence dogs' final performance. Since dogs were performing during *extinction* during test trials (dogs were not provided any feedback on any samples), we could use dogs' time at port to assess their certainty about sample categories.

Behavioral analysis

Video data were obtained for every training and testing session in this study. Each video was filmed at 60 frames per second. These data were coded using BORIS (Friard and Gamba, 2016). BORIS is a free behavioral coding software. One coder coded all of the data, and a second coder coded a subset of the data for inter-rater reliability. When the IRR was determined to be acceptable between the main coder and the second coder, the main coder's data were used for the analysis.

Supplementary Table 1 contains the ethogram used for each video. The coders were unaware of the location of the positive and negative samples. The coders had to make judgments about the start and end of each behavior (dogs' duration at port was calculated as the time at which their nose was within 4 in. of the port, and this could be difficult to ascertain sometimes depending on the video angle). As such, this introduced some variability into the timing data.

An inter-rater analysis was run in R to determine the reliability of coders for four randomly chosen videos out of the 12 total test videos (30%). Duration of time spent at port was compared between coders. The average duration difference between the two coders was 0.17 seconds. The inter-rater analysis was run based on a single-rating, consistency, one-way mixed-effect model. This model shows that the intraclass coefficient was 0.77, with a confidence interval of 0.678-0.835. This is considered good reliability (Koo and Li, 2016).

In addition, a different experienced research assistant who did not assist with the SNIP study but was familiar with the dogs and their general searching behavior in the wheel room was asked to watch the videos of the SNIP test sessions and mark when these dogs showed any change in behavior at a sample. This was a binary coding task in which the dogs were marked as either showing change in

behavior at sample or not showing change in behavior at sample. The coder was instructed that "change in behavior" included both the dogs' trained final behaviors and any behaviors indicating interest in a sample (e.g., pawing at the sample, hovering over the sample, and hesitating at the sample). They were also told, conversely, that a "pass" at a sample (no change in behavior) consisted of sniffing and leaving a sample within 0.5 seconds.

A second coder (another research assistant who did not assist with the SNIP study but was familiar with the dogs' behaviors) recoded two videos out of the total set of videos. The inter-rater analysis was run based on a single-rating, accuracy, one-way mixed-effect model. This model shows that the intraclass coefficient was 0.96, with a confidence interval of 0.942-0.973. This is considered excellent reliability (Koo and Li, 2016).

Statistical models

Models were done in R version 4.2.0.

Model 1. Effects of Sample Type and presence of SNIP on duration at odor.

This linear mixed-effect model assessed the effects of Sample Type, and presence of SNIP on the duration of time dogs spent at each novel sample. Assumptions were tested for the use of a linear mixed-effect model, and visual examination of the residuals of the analysis was not normally distributed. To address this, a logarithmic transform of duration data was taken to ensure normalization and linearity of residuals. In addition, an outlier analysis was performed using Rosner's test, and no outliers were found in these data.

Sample Type (blood plasma or nasal secretion) and Sample Status (SNIP-positive or SNIP-negative) were fixed effects, and dog-tested (Crunch, Kodi, Pacy, or Xoxo) and number of test (1, 2, or 3) were random effects. The log-transformed duration at odor was used as the outcome variable.

Pairwise comparisons for Sample Type and Sample Status were done using the emmeans package and adjusted using a Tukey correction.

Model 2. Effects of Sample Type and presence of SNIP on trained final alert at odor.

A model was generated to assess the effects of Sample Type and presence of SNIP on the presence of dogs' trained final alert at each novel sample. A binomial linear mixed-effect model was attempted with dog and number of the test as random effects, but this model did not converge. As such, a binomial generalized linear model was used instead with Sample Type and presence of SNIP as independent variables and alert presence as the dependent variable.

Model 3. Effects of Sample Type and presence of SNIP on change in behavior at odor.

While dogs should perform their trained final alert on a sample they identify as positive, Dechant suggested that dogs may not exhibit their trained final response if there are any changes in the search context or other disruptors to the search conditions (Dechant, 2021). In this case, dogs were performing in extinction (performing a previously learned behavior without the reward that has been previously associated with that behavior), which they had never done before. They were also examining samples in a novel medium. As such, dogs may exhibit a different, more subtle change in behavior than their full trained final response. Dogs also may not know what is expected of them when the testing scenario changes. A model was generated to assess the effects of Sample Type and presence of SNIP on the presence of dogs' change in behavior at each novel sample. A binomial linear mixed-effect model was attempted with dog and number of the test as random effects, but this model did not converge. As such, a binomial generalized linear model was used instead with Sample Type and presence of SNIP as independent variables and alert presence as the dependent variable.

Results

Effects of sample type and presence of SNIP on duration at novel odor

There was no significant effect of Sample Type on duration at port ($\beta_1(66) = -0.19$, $t = -1.87$, $P = 0.06$), but significant effects were found as a result of SNIP presence ($\beta_1(66) = 0.32$, $t = 3.34$, $P = 0.001$). In addition, an interaction between Sample Type and SNIP presence was found ($\beta_1(66) = 0.33$, $t = 2.15$, $P = 0.06$). Dogs spent an average of 3.73 seconds at positive ports, and an average of 1.00 seconds at negative ports (see Figure 3).

Using the emmeans package (Lenth et al., 2022), post hoc comparisons were done to examine contrasts between Sample Type and Sample Status. These comparisons are found in Table 3. Notably, there are significant differences between duration at port for positive plasma (average duration = 4.45 s) and negative plasma (average duration = 0.78 s) as well as positive nasal (average duration = 3.01 s)

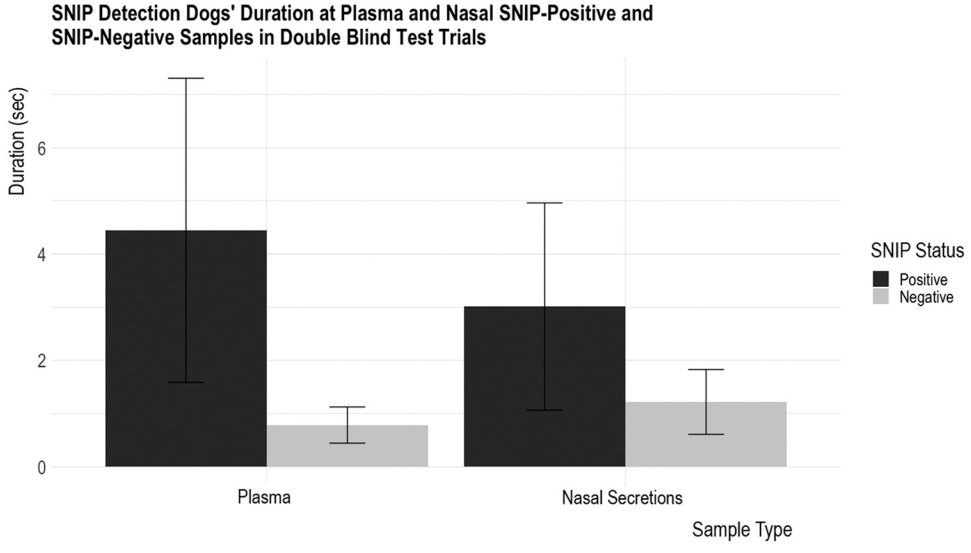

Figure 3. A graph of dogs' duration of time spent at samples (seconds) as a function of the Sample Status (positive or negative for SNIP) and Sample Type (plasma or nasal).

Table 3
Post hoc contrasts between Sample Type and Sample Status for Model 1 analysis.
This table shows the results of a post hoc contrast analysis done using the emmeans package in R (Lenth et al., 2022). Individual contrasts examining the effect of Sample Type and Sample Status on duration at sample are shown.

Contrast	t-value	*P*-value
Positive nasal-negative nasal	−3.337	0.007
Positive nasal-negative plasma	5.092	< 0.0001
Positive plasma-negative plasma	−5.404	< 0.0001
Positive plasma-negative nasal	−3.951	0.001
Negative nasal-negative plasma	1.875	0.249
Positive nasal-positive plasma	−1.226	0.613

Table 4
Post hoc contrasts between Sample Type and Sample Status for Model 2.
This table shows the results of a post hoc contrast analysis done using the emmeans package in R (Lenth et al., 2022). Individual contrasts examining the effect of Sample Type and Sample Status on trained final alert are shown. *P*-values less than 0.05 are marked with an asterisk (*).

Contrast	z-value	*P*-value
Positive nasal-negative nasal	−1.60	0.39
Positive nasal-negative plasma	2.24	0.11
Positive plasma-negative plasma	−2.82	0.02*
Positive plasma-negative nasal	−2.43	0.07
Negative nasal-negative plasma	1.19	0.63
Positive nasal-positive plasma	−1.17	0.64

and negative nasal (average duration = 1.22 s). This demonstrates that within each Sample Type, dogs demonstrate longer alert times at the SNIP-positive samples than the SNIP-negative samples. However, the interaction between Sample Type and Sample Status in Model 1 shows that dogs' performance was different in the plasma and nasal samples. While a significant effect of SNIP status was found on time duration within both the plasma and nasal mediums, the average time spent at positive nasal samples was less than the average time spent at positive plasma samples. In addition, dogs spent more time at negative nasal samples than they did at negative plasma samples. The difference in duration in contrasts was not significant; however, post hoc comparisons showed no significant difference between duration at positive nasal (avg duration = 3.01) and positive plasma (avg duration = 4.45), or negative nasal (avg duration = 1.22) and negative plasma (avg duration = 0.78).

Effects of sample type and presence of SNIP on trained final alert presence at novel odor

There was no significant main effect of Sample Type, z = −1.19, *P* = 0.23. There was no significant main effect of Sample Status, z = 1.59, *P* = 0.11. There was no interaction, z = 1.63, *P* = 0.10. A table of post hoc contrasts examines the interaction of the levels of these variables (Table 4). We find that there was a significant difference between alerts on positive plasma and alerts on negative plasma, z = −2.82, *P* = 0.02. There was no significant difference, however, between alerts on positive nasal secretions and alerts on negative nasal secretions, z = −1.59, *P* = 0.38. Figure 4 shows the proportion of alerts on positive and negative plasma and nasal samples.

Effects of sample type and presence of SNIP on change in behavior at novel odor

This analysis examined the presence of any change in behavior on a sample. There was no significant main effect of Sample Type, z = −0.48, *P* = 0.63. There was a significant main effect of Sample Status, z = 3.30, *P* = 0.001, where dogs alert more on SNIP-positive samples (28 alerts out of 36 total positive samples) than SNIP-negative samples (10 alerts out of 45 samples). There was no significant interaction between Sample Type and Sample Status, z = 0.74, *P* = 0.46. Figure 4 shows dogs' alert count as a function of Sample Type and Sample Status. Table 5 shows post hoc contrasts between Sample Type and Sample Status. Figure 5.

Discussion

This study examined dogs' ability to detect the olfactory signature of SNIP in blood plasma and nasal secretions. Dogs were trained on SNIP-positive and SNIP-negative blood plasma and then tested on novel SNIP-positive and SNIP-negative blood plasma and nasal secretions. Dogs were able to detect SNIP presence in novel blood plasma samples. While dogs did not perform their trained final response significantly more often at novel SNIP-positive nasal secretions than SNIP-negative nasal secretions, they did show a

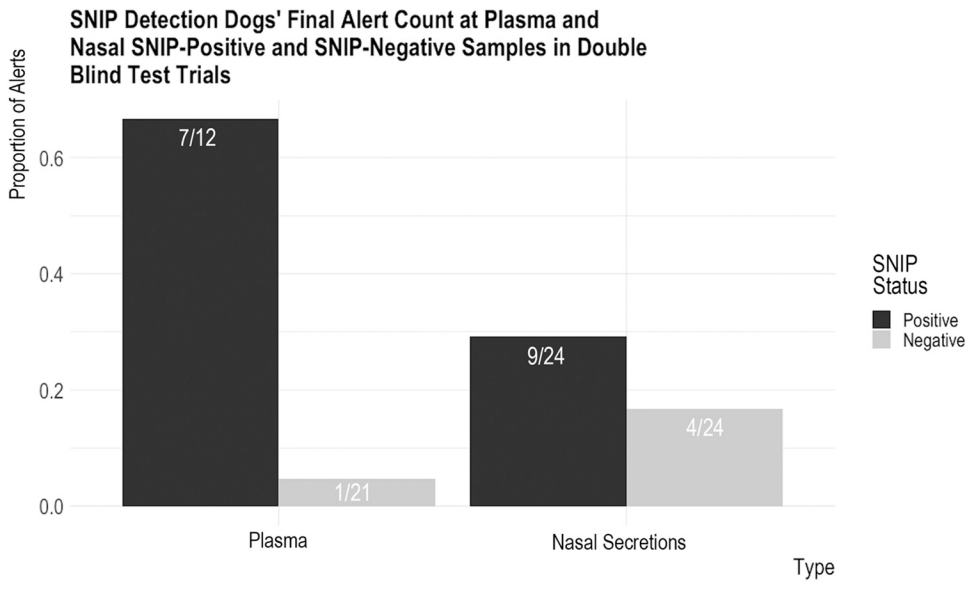

Figure 4. A graph of the proportion of trained final alerts at sample as a function of the Sample Status (positive or negative for SNIP) and Sample Type (plasma or nasal).

Table 5
Post hoc contrasts between Sample Type and Sample Status for change in behavior analysis.

This table shows the results of a post hoc contrasts analysis done using the emmeans package in R (Lenth et al., 2022). Individual contrasts examining the effect of Sample Type and Sample Status on change in behavior at sample are shown. *P*-values less than 0.05 are marked with an asterisk (*).

Contrast	z-value	*P*-value
Positive nasal-negative nasal	−3.34	0.007*
Positive nasal-negative plasma	5.10	< 0.0001*
Positive plasma-negative plasma	−5.40	< 0.0001*
Positive plasma-negative nasal	−3.95	0.001*
Negative nasal-negative plasma	1.88	0.25
Positive nasal-positive plasma	−1.23	0.61

change in behavior significantly more often at novel SNIP-positive nasal samples than SNIP-negative nasal samples, despite never having been presented with nasal secretions before in training. This suggests that the SNIP-positive nasal secretions and blood plasma do share common properties.

While there is published evidence that dogs are capable of generalizing from singular odors to more complex compounds (Lazarowski and Dorman, 2014; Moser et al., 2019), limited literature in the medical detection domain discusses generalization between two complex compounds, and there is no prior use of nasal secretions as an olfactory medium. This study provides evidence that it is possible to train dogs to detect an olfactory signature of positive SNIP in blood plasma; furthermore, dogs can be used to determine similarities between odor mediums if criteria for trained final alert are lowered to a change in behavior. Noninvasive early detection of SNIP recurrence allows for more personalized care to be applied when deciding how aggressively to pursue abnormal findings on imaging or exam and negative biopsies. It may also help improve SNIP surveillance schedules postoperatively.

Dogs successfully differentiated the SNIP-positive blood plasma odor from SNIP-negative blood plasma. This finding was consistent with the reported unique VOC signature differentiating SNIP in blood plasma from healthy patients (Chaskes et al., 2022), and with several reports of dogs detecting other unique VOC signatures of cancer (Moser and McCulloch, 2010) and disease (Taylor et al., 2018; Angeletti et al., 2021).

Dogs learned the SNIP blood plasma odor signature despite the limited number of training samples available. While more training samples generally facilitate optimal generalization and specificity, samples from patients with particular diseases are often difficult to obtain. This limitation is common in canine detection studies, as researchers are often interested in training dogs to detect diseases that are difficult to diagnose. As such, small sample sets are used out of necessity. However, using small training sets runs the risk of dogs memorizing the training set and failing to generalize to novel items (Elliker et al., 2014; Essler et al., 2021b). The ideal number of training samples to optimize specificity and sensitivity depends on several factors (Moser et al., 2019), including the complexity of the target odor, the experience of the dogs used in the study, the number of times each sample will be used in a training session (Elliker et al., 2014; Essler et al., 2021b), and the similarity of the training odor to the testing odor. Given the number of factors involved, it can be difficult to identify the minimum number of samples needed for ideal generalization. For example, dogs have succeeded in identifying the presence of accelerants in a double-blind test after experience with just 20 positive training samples and 20 negative training samples (Wright et al., 2017); however, in another study, dogs failed to differentiate prostate cancer urine samples from control urine samples in a test despite training with 50 positive samples and 67 controls (Elliker et al., 2014).

Dogs trained only on plasma samples demonstrated a change in behavior significantly more often on SNIP-positive nasal secretions than SNIP-negative nasal secretions; this suggests that there may be a commonality in the SNIP signature across mediums. Dogs have previously generalized disease odor from saliva to sweat and urine (Jendrny et al., 2021), from cell lines to blood plasma (Murarka et al., 2019), and from urine to saliva (Essler et al., 2021b), but there are no current publications that document odor generalization from blood plasma to nasal secretions. Furthermore, the dogs in this study were able to generalize odor without additional training using nasal secretion samples, which suggests that the odor learned from the SNIP-positive blood plasma shares features with the odor from the SNIP-positive nasal secretions. This is the first study in which dogs have been tested on nasal secretions; the change in behavior that

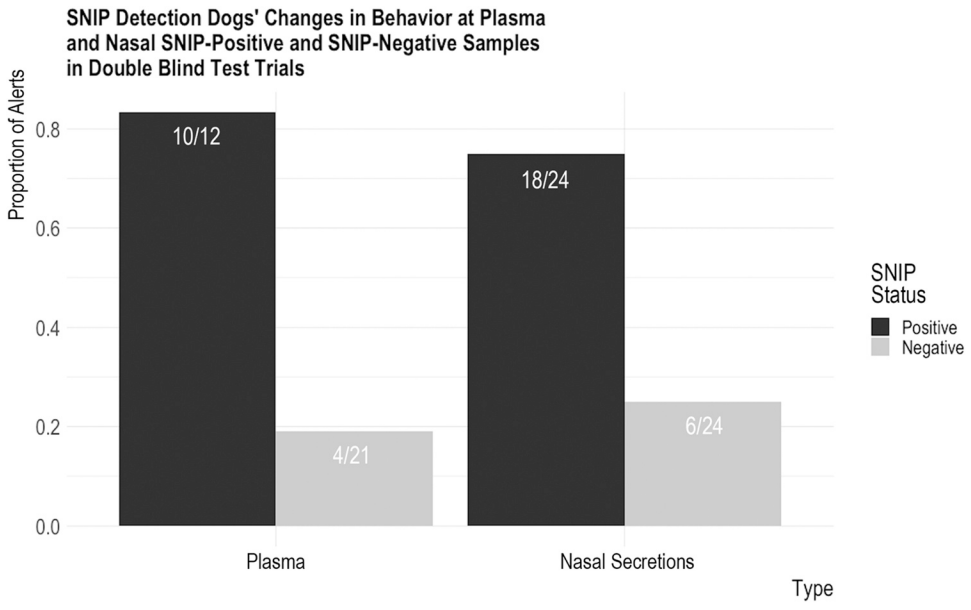

Figure 5. A graph of the proportion of change in behavior at sample as a function of the Sample Status (positive or negative for SNIP) and Sample Type (plasma or nasal).

dogs demonstrate is promising, and suggests that dogs trained on nasal secretions specifically could learn this odor, which is encouraging for the future of noninvasive diagnostic techniques. Nasal secretions contain biological markers that provide information about local inflammation from which they were collected, and as such are practical and readily obtainable indicators of nasal health. They are also easily obtained with relatively low morbidity. The data provided here suggest that the VOC analysis of nasal secretions could lead to diagnostic practices that both identify the disease earlier in its development and require less invasive sample collection techniques.

This study used dogs with a range of prior detection experience. While we did not test enough samples to be able to compare performance between dogs, this would be an important further step to help determine whether prior experience with odor leads to improved odor learning in future trained scents. In addition, all of our dogs were trained initially with the errorless learning method, but it is unclear whether this method of instruction benefits all dogs. The use of an additional concentration cue may be better suited for dogs newer to odor learning, as dogs with more experience may overly rely on this concentration cue and fail to learn the target odor.

Since the conclusion of this study, the PVWDC has also begun utilizing a different blind testing technique than the one presented in this paper to improve canine test performance. The approach used here kept handlers blinded for the entire test trial and did not provide any reward for dogs' correct alerts. Anecdotally, dogs in this study who were especially sensitive to handler feedback seemed to exhibit more reluctance to alert to novel odors as their testing session progressed. It was thought that this was a direct result of the lack of instantaneous feedback provided to the dog after exhibiting a positive alert. Our new approach to testing allows handlers to remain blinded while the dog is engaged in a trial until the dog correctly alerts to a positive sample, at which point a light provides feedback to the handler from researchers who remain out of the room while the dog is searching. This allows the now unblinded handler to deliver positive reinforcement for a correct alert, which could likely improve the resilience and confidence of dogs throughout the testing phase of a study. It would be advantageous — for this study's results as well as for future scent detection studies — to investigate dogs' performance and response to increased feedback in the blind testing phase.

Future studies should also examine the benefits and drawbacks of training on larger numbers of positive and negative samples as well as more diverse sample sets. Increasing the biological variability by including a larger number of unique positive and negative samples would provide evidence that dogs are abstracting a unique SNIP odor category from the samples, regardless of their demographic features. Currently, we can only conclude that there is a similarity in SNIP signature across SNIP plasma and nasal secretions based on dogs' change in behavior responses at positive and negative samples. A quantitative comparison of test trials between the dogs trained on this sample set and dogs trained on an extended sample inventory could also provide insight as to how the ability to generalize a target disease odor across biological mediums is affected by inventory size and variation, if at all.

Last, it would be advantageous from both a scent detection training and diagnostic perspective to examine how canine olfaction and GC–MS techniques could be utilized simultaneously to garner further information on the VOC profile of SNIP. Ideally, future studies would include the use of both canine olfaction and GC–MS techniques. By utilizing both approaches concurrently, VOC identification within LDA models could be improved, and the types of samples presented to the dogs and the way they are presented could be continuously informed throughout training by the quantitative VOC data.

Conclusions

This study demonstrates the viability of training dogs to detect SNIP within blood plasma. In addition, it demonstrates similarity between the signature SNIP disease odor in one trained medium, blood plasma, and a novel medium, nasal secretions. Together, this provides evidence for a universal SNIP odor signature shared between blood plasma and nasal secretions.

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Conflict of Interest

The authors declare no conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jveb.2023.02.008.

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