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A new detection method for canine and feline cancer using the olfactory system of nematodes

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ABSTRACT

Cancer is the leading cause of death in dogs and cats. Early diagnosis of cancer is critical for effective treatment and improving survival rates. Nematode-NOSE (N-NOSE) is a commercially available non-invasive human cancer screening test that uses the sense of smell of the nematode *Caenorhabditis elegans* showing a distinct chemotactic response toward the urine of an individual with cancer compared to healthy ones. 15 types of human cancer (stomach, colon-rectum, lung, breast, pancreas, liver, prostate, uterus, esophagus, gallbladder, bile duct, kidney, urinary bladder, ovary, and oropharynx cancers) can be detected by N-NOSE.

A non-invasive method for accurate cancer screening is needed for pets. In this study, we evaluated the effectiveness of N-NOSE in detecting cancer using canine and feline urine samples. We found a significant difference in chemotaxis index values between healthy subjects and cancer patients in both canine ($p < 0.01*$) and feline ($p < 0.04*$) urine samples. Receiver operating characteristic (ROC) analysis highlights the good performance of the test with areas under the curve (AUC) of 0.8114 and 0.7851 for dogs and 0.7667 and 0.9000 for cats when using 2 different dilutions of urine samples. Our study suggests that N-NOSE has the potential as a simple, accurate, and low-cost cancer screening test in both dogs and cats.

1. Introduction

Cancer is a major cause of death in both companion animals and humans. In the United States, of 76.8 million domesticated dogs and 58.4 million cats, 4 million dogs and 4 million cats are diagnosed with cancer each year [1,2]. By comparison, over 1.6 million humans have been diagnosed with cancer annually in the USA [2]. In Japan, the number of domesticated dogs and cats stands at 8.5 million and 9.6 million, respectively [3]. An epidemiological survey in Japan showed that neoplastic diseases are diagnosticated on 9.6% of dogs (0.8 million dogs) and 5.6% of cats (0.5 million cats) [4]. An early cancer diagnosis is of paramount importance to enhance anti-cancer treatments and improve survival rates along with overall prognoses [5]. Currently, early-stage cancer may be detected by computed tomography (CT) and magnetic resonance imaging (MRI). Still, the use of these techniques is precluded by their high cost and requirement for general anesthesia. Therefore, a simple, non-invasive examination is needed to detect

early-stage cancer with less economic burden and less physical stress on animals.

Non-invasive cancer detection using body fluids such as urine to detect volatile biomarkers is a fast-growing research field, from using analytical techniques to exploiting living organisms such as nematodes or even ants [6–9]. It relates to the cancer smell, the end-product of metabolic changes producing patterns of volatile organic compounds (VOCs) observed in cancer [10–12]. The volatilome in urine is increasingly being studied, and several VOCs biomarkers have been identified for several cancers [13]. For instance, VOCs that are potentially specific biomarkers for breast cancer (bicyclo [2.2.1]heptane, 7,7-dimethyl-2--methylene, farnesene, caryophyllene, D-limonene, pinocarvone, himachalol, himachalane, bisabolol, and bisabolene) have been identified [14]. Interestingly, most of these VOCs are involved in the biosynthesis of the terpenoids [14]. In lung cancers, 2-butanone, 1-propanol, isoprene, ethylbenzene, styrene, and hexanal are the VOCs that appear to be specific biomarkers in breath samples [15]. Dogs trained with

olfactory associative learning can recognize specific cancer smell in urine to detect human lung cancer and breast cancer [16–19]. Hirotsu et al. were the first to demonstrate that the nematode *Caenorhabditis elegans* is attracted to urine from cancer patients but tends to avoid urine from healthy persons [20]. *C. elegans* has a refined sense of smell that can be utilized as a very powerful sensor for a cheap and non-invasive cancer detection method using urine samples [7,8,20–25].

C. elegans has ~1200 olfactory receptor-like genes and approaches a preferred odor and move away from a disliked odor [26]. The excellent olfaction of *C. elegans* is the product of sensory neurons AWA, AWB, and AWC that are well known to accept volatile substances [20,26–28]. AWA and AWC neurons cause attractive behavior, while the AWB neuron mediates repellent behavior [20,26–28]. ODR-3 (G protein α) functions as a major component of the sensory signaling pathways of AWA and AWC neurons in response to volatile substances but not to water-soluble substances [20,29]. Interestingly, the *odr-3* mutant nematode was not attracted to the culture supernatant of human cancer cells, suggesting that *C. elegans* responds to cancer cell-specific volatile substances via ODR-3 [28].

Nematode-NOSE (N-NOSE), commercially available in Japan (www.hbio.jp/en/), is a cancer screening test that is based on the chemotactic characteristics of *C. elegans* (Fig. 1). *C. elegans* shows avoidance of the urine of healthy individuals while displaying a chemotactic attraction toward the urine of patients with 15 types of cancer (stomach, colon-rectum, lung, breast, pancreas, liver, prostate, uterus, esophagus, gallbladder, bile duct, kidney, urinary bladder, ovary, oropharynx) rendering N-NOSE a primary multi-cancer screening test [20,24,25,30,31]. N-NOSE clinical studies with human patients from cancer stages 0 to IV have highlighted the good performance at early detection (stages 0-I) without lymphatic metastasis, in addition to detecting advanced stages [20,24,25,30,31]. This test only requires ≥1 mL of urine, which is non-invasive, painless, and stress-free for the subject [20,28]. N2 wild-type *C. elegans* nematodes used in N-NOSE are hermaphroditic, can easily propagate on agar while consuming *E. coli.*, maintain a stable genetic homogeneity, and importantly, do not need to be trained for

chemotactic response with urine [20,28]. For reference, N-NOSE for humans costs ~ USD $114 per test.

Based on comparative oncology between human and feline & canine species, we hypothesized that *C. elegans* might show a distinct chemotaxis response between healthy and cancer urine in both cats and dogs. The behavioral chemotactic response of *C. elegans* to odor is concentration-dependent [20]. Previously, we showed that the chemotactic response of *C. elegans* toward human urine is optimal at 3 dilutions (10^{-1}, 10^{-2}, 10^{-3}) [20,31]. Therefore, we hypothesized the same might be observed with canine and feline urine samples. To investigate whether *C. elegans* detects and distinguishes odors of canine and feline urine from cancer patients and healthy subjects, we conducted a pilot clinical study with 37 canines (19 healthy and 18 with tumor) and 23 felines (10 healthy and 13 with tumor) (Table 1).

2. Materials and methods

2.1. Study population and ethics

Urine samples from dogs and cats were obtained from Matsuiyamate Animal Hospital (Kyoto, Japan) or other animal hospitals and breeders affiliated with Anicom Specialty Medical Institute Inc. (Tokyo, Japan). The study population included 37 canines (19 healthy and 18 diagnosed with tumor) and 23 felines (10 healthy and 13 with tumor) (Tables 1A and 1B). The age, race, and sex of animals are indicated in Table 1. Cancer patients were diagnosed with benign or malignant tumors in various locations by histologic examination, cytology, MRI, CT, or chest radiography (Table 1). The study was approved by the Ethics Committee of the Matsuiyamate Animal Hospital, and Anicom Specialty Medical Institute Inc. Clinical examinations were performed according to the principles of the WMA Declaration of Helsinki. Informed written consent was obtained from the owners for each participant before study entry.
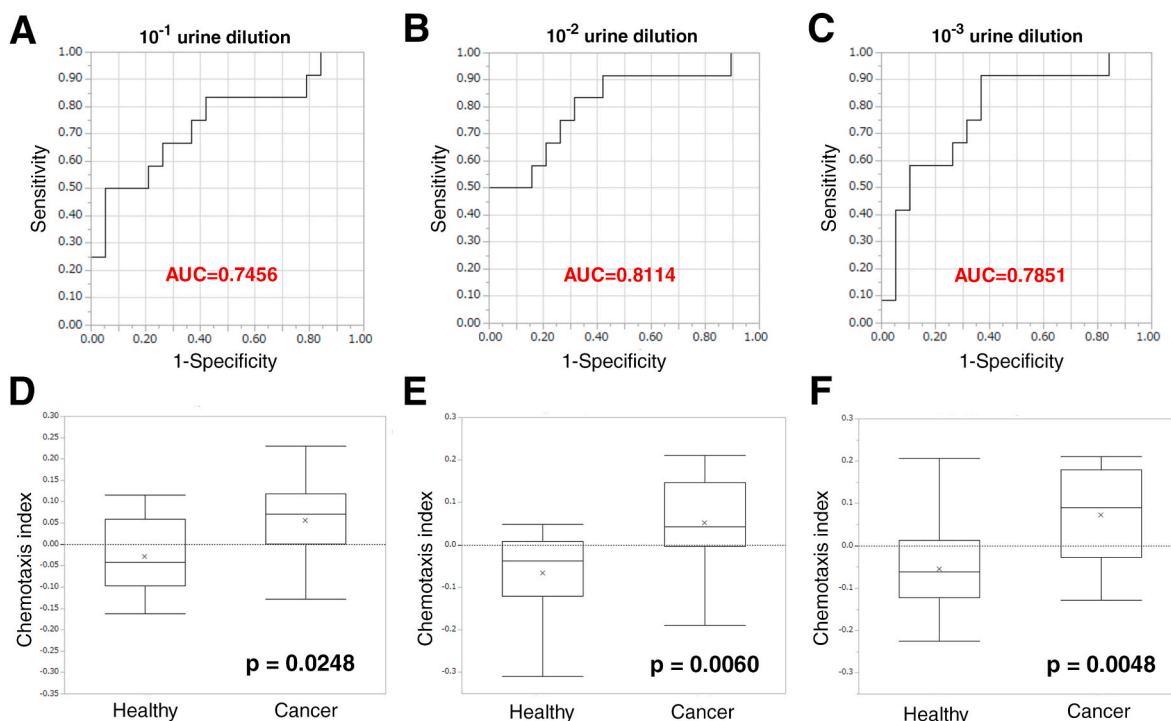

Fig. 1. N-NOSE assay results (malignant vs healthy) for diluted canine urine samples. A-C: Area under the curve (AUC) values at **A)** 10^{-1}, **B)** 10^{-2}, **C)** 10^{-3} dilution determined by receiver operating characteristic analysis. **D-F**: Box plot of chemotaxis index of *C. elegans* to canine urine samples diluted at **D)** 10^{-1}, **E)** 10^{-2}, or **F)** 10^{-3} from 19 healthy dogs and 12 dogs with cancer. Error bars = SEM (*n* ≥ 3 assays for all samples).

Table 1
Diagnostic records of urine samples used in the N-NOSE study.

A: Canines

Subject	Breed	Sex	Age	Cancer treatment	Tumor type	Tumor site	Benign or malignant	Diagnostic method
20	Miniature Dachshund	F	11	UC	Benign hepatic tumor (suspected)	Liver	B	HE
21	Welsh Corgi	M	12	UC	Unknown cancer	Brain	U	MRI
22	Miniature Dachshund	F	14	UC	Urothelial carcinoma	Urethra	M	HE
23	Miniature Dachshund	M	14	UC	Bone tumor due to enlargement of bone tissue	Lumbar bone	B	CE
24	Miniature Dachshund	F	13	UC	Hepatocellular carcinoma	Liver	M	HE
25	Miniature Dachshund	F	10	UC	Benign mixed tumor	Mammary gland	B	HE
26	Border Collie	M	11	UC	Undefined cancer	Liver	U	CT
27	Miniature Dachshund	F	13	UC	Undefined cancer	Mammary gland	M	HE
28	Miniature Dachshund	F	13	UC	Benign tumor	Mammary gland	B	HE
29	Miniature Dachshund	F	14	UC	Undifferentiated sarcoma	Jejunum	M	HE
30	Beagle	M	13	UCT	Large granular lymphocyte-lymphoma	Liver, Spleen	M	CE
31	Pomeranian	M	15	UCT	Poorly differentiated lymphoma	Lymph node	M	CE
32	French bulldog	F	11	UCT	Lymphoma	Multicentric lymph node	M	HE
33	Maltese	F	10	UCT	Undefined cancer	Thyroid	M	CE
34	Miniature Dachshund	M	16	UCT	Urothelial carcinoma	Bladder	M	HE
35	Welsh Corgi	M	12	UC	Multiple myeloma, cutaneous lymphoma	Skin	M	HE
36	Bernese Mountain Dog	M	12	UC	GI lymphoma	Gastrointestinal tract	M	HE
37	Bernese Mountain Dog	M	12	ASR	GI lymphoma	Gastrointestinal tract	M	HE

B: Felines

Subject	Breed	Sex	Age	Cancer treatment	Tumor type	Tumor site	Benign or malignant	Diagnostic method
11	American Shorthair	M	16	UC	Undefined cancer	Lung	M	CE
12	Mix	F	16	UC	Undefined cancer	Mammary gland	M	CE
13	Mix	M	15	UC	Adenocarcinoma	Nasal cavity	M	CE
14	Persian	F	17	UC	Lymphoma	Unknown	M	CE
15	Mix	F	14	UCT	Mammary carcinoma	Mammary gland, lung metastasis	M	CE
16	British shorthair	M	1	UCT	Lymphoma	Unknown	M	CE
17	Mix	F	14	UCT	Renal lymphoma	Kidney, lung metastasis	M	CE
18	Mix	F	14	UCT	Unknown cancer	Mammary gland	M	HE
19	Mix	F	3	UCT	Lymphoma	Intrathoracic lymph nodes	M	Pleural effusion smear, X-ray
20	American Shorthair	F	10	UC	Adenocarcinoma	Lung	M	CT, FNA
21	Somali	M	10	UCT	GI lymphoma	Gastrointestinal tract	M	HE
22	Mix	F	8	UCT	Lymphoma	Lymph node	M	HE
23	Mix	F	9	ASR	Adenoma	Skin	B	CE

Legend to Table 1: F = female; M = male; ASR = after surgical resection; UC = Untreated cancer; UCT = Under cancer treatment; GI = Gastrointestinal; B = Benign; Ma = Malignant; CT = Computed tomography; MRI = Magnetic resonance imaging; FNA = Fine needle aspiration; HE = Histologic examination; CE = Cytologic examination; Canines subjects 1 to 19 and felines subjects 1 to 10 are healthy subjects, so the description in the table is omitted.

2.2. Urine sample collection

The samples were obtained from healthy subjects by spontaneous urination. Catheterized samples were collected from cancer patients. Urine samples were visually confirmed to have no obvious abnormalities, such as turbidity, and then stored at −20 °C within 30 min of collection.

2.3. Nematode culture

C. elegans N2 strain (wild-type) were cultured on a 10 mL nematode growth media (NGM) agar plates (1.7% Bacto agar, 0.25% Bacto peptone, 0.3% NaCl, 25 mM KPO$_4$ buffer pH 6.0, 1 mM CaCl$_2$, 1 mM MgSO$_4$, and 5 μg/mL cholesterol) seeded with *E. coli* as a food source [20,27,28,32–34].

2.4. Measurement of N-NOSE

N-NOSE assays with canine or feline urine samples were performed using the same protocol we described for human urine samples [20,24, 25,28,31]. Briefly, for chemotaxis assays, we printed five marks on the bottom of a 9-cm assay plate (2.0% Bacto agar, 5 mM KPO$_4$ buffer pH 6.0, 1 mM CaCl$_2$, 1 mM MgSO$_4$): 3-mm diameter circle in the center, 2 + marks 35 mm from the center on the left side, and 2 ● marks 35 mm

from the center on the right side. The distance between the + marks (and the ● marks) is 25 mm, and the different marks are symmetric to the plate center. First, 0.5 μL of 1 M NaN$_3$, which has an anesthetic effect on nematodes, was placed with + and ●, respectively. After diluting each urine sample 10, 100, and 1000 times with Milli-Q water, 1 μL of diluted sample was spotted on the + marks. Subsequently, 50–100 synchronized young adult N2 worms were placed above the center circle of the assay plate. After 30 min of free-roaming, the worms in the attraction area (A) on the sample side and in the avoidance area (B) were counted (Fig. S1A). The chemotaxis index was calculated as (A - B)/(A + B) (Fig. S1). In this study, the N-NOSE index cut-off is 0. A negative value (−1 to 0) indicates repulsion from the urine sample; a positive value (>0) indicates attraction to the urine sample (Fig. S1B) [20,27,28,33]. Analysis of N-NOSE data for humans has indicated that false negatives may occur due to problems with urine samples such as contamination, sample processing problem, sample transport, and cold storage issue. Importantly, whether another substance in the urine or other disease may trigger false negatives have not been found in clinical studies with human urine. The effect of the subject's background on N-NOSE has been investigated in human clinical studies, and only the presence/absence of cancer correlates with N-NOSE [23].

2.5. Statistical analyses

Statistical analyses were performed using JMP14 (SAS Institute). A Welch *t*-test was used for comparison between groups. For all analyses, a probability value of $p \leq 0.05$ was considered statistically significant. The areas under the curve (AUC) values were calculated using the receiver operating characteristic (ROC) analysis [24,25]. In this study, "malignant" refers to malignant tumors only.

3. Results and discussion

We conducted a pilot clinical study with 37 canines (19 healthy and 18 cancer subjects) and 23 felines (10 healthy and 13 cancer subjects) (Tables 1A and 1B). For both canine and feline study populations, significant differences were observed in chemotaxis indexes between healthy subjects and cancer patients. Considering the tumor population (subject population with benign and malignant tumors) versus healthy, at three urine dilutions (10^{-1}; 10^{-2}; 10^{-3}), *p*-values for canines are: 0.1348 (10^{-1} dilution of urine); 0.0144* (10^{-2}) and 0.0172* (10^{-3}); *p*-values for felines are: 0.6538 (10^{-1}); 0.0216* (10^{-2}) and 0.0008* (10^{-3}).

Focusing on the malignant versus healthy population, for canine samples, the Welch's *t*-test indicated that the chemotaxis index was significantly different between healthy and cancer groups when canine urine was diluted at 10^{-2} ($p = 0.0060*$) or 10^{-3} ($p = 0.0048*$) (Fig. 1E–F) and Table 2A); the dissimilarity was significant less distinctive at the 10^{-1} dilution ($p = 0.0248*$) (Fig. 1D and Table 2A).

Table 2
Comparison of *C. elegans* chemotactic index values for healthy urine and malignant tumor urine.

A: Canines					
Sample dilution	Total samples	Healthy dogs	Cancer dogs	*p* value: Welch *t*-test	AUC: ROC analysis
10^{-1}	31	19	12	0.0248*	0.7456
10^{-2}	31	19	12	0.0060*	0.8114
10^{-3}	31	19	12	0.0048*	0.7851
B: Felines					
Sample dilution	Total samples	Healthy cats	Cancer cats	*p* value: Welch *t*-test	AUC: ROC analysis
10^{-1}	22	10	12	0.7342	0.4833
10^{-2}	22	10	12	0.0367*	0.7667
10^{-3}	22	10	12	0.0007*	0.9000

The ROC analysis at 10^{-2} and 10^{-3} dilutions suggests that N-NOSE has high AUC values (0.8114 for 10^{-2}, 0.7851 for 10^{-3}) for canine cancers highlighting the ability to distinguish between cancer patients and the healthy group (Fig. 1B–C) (Table 2A).

Likewise, for feline urine samples, malignant versus healthy groups, the chemotaxis index was significantly different between healthy subjects and cancer patients at dilutions of 10^{-2} ($p = 0.0367*$) or 10^{-3} ($p = 0.0007*$) (Fig. 2 E-F and Table 2B); there was no significant difference at 10^{-1} ($p = 0.7342$) (Fig. 2D and Table 2B). In addition, ROC analysis showed high discrimination performance between healthy and cancer subjects, with the AUC values of 0.7667 for 10^{-2} and 0.9000 for 10^{-3} urine dilution (Fig. 2B–C and Table 2B). Despite limitations due to the size of this pilot study, no difference was observed in the chemotactic index between healthy urine and urine for benign tumors.

In this study, *C. elegans* was attracted to urine samples from both canine and feline cancer patients. N-NOSE chemotaxis indexes were significantly different between the healthy and cancer groups, as seen for humans [20,23–25,31,35]. Comparing the healthy versus malignant groups, the canine group shows a greater significant difference in all urine sample concentrations 10^{-1}, 10^{-2}, and 10^{-3}, compared to the feline study group (Tables 2A and B). Notably, the N-NOSE chemotaxis index was not affected by sex or age (Figs. S2 and S3).

Unexpectedly, all 3 urine concentrations of a 6-year-old healthy cat were strongly repellent. For cancer patients, cat 16 (1-year-old) and cat 19 (3-year-old) showed an attraction behavior at sample concentrations of 10^{-2} and/or 10^{-3}. Dogs 30, 31, and 34 with malignant cancers received prednisolone and ursodeoxycholic acid treatment at the time of sample collection; *C. elegans* showed attraction behavior at all 3 concentrations, and these two medications did not affect the assay (Fig. S4). *C. elegans* showed a repellent behavior at all 3 urine concentrations from dog 13. However, for malignant cancers, subjects 30, 31, and 34, *C. elegans* showed attracting behavior at all three concentrations. Taken together, both prednisolone and ursodeoxycholic acid medications do not appear to affect chemotaxis assays (Fig. S4).

In human, a 10^{-1} urine dilution allows *C. elegans* to have statistically significantly different chemotaxis assay result between cancer and healthy groups [20]. However, it is not the case in both canine and feline urine samples, where urine must be further diluted to 10^{-2} and/or 10^{-3} (Figs. 1 and 2). It is unclear why canine and feline urine needs to be diluted more than human urine. A study of a mouse model of pancreatic tumors showed very accurate results with a 10^{-3} dilution; therefore, species intrinsic differences may explain these discrepancies in dilution concentrations [31]. In addition, it is estimated that the larger the tumor size relative to the body size, the higher the odor concentration in urine. Although we analyzed cancer patient urine samples from twelve small-breed dogs (nine Miniature Dachshunds, one Pomeranian, one Maltese, one French Bulldog), four medium-sized breeds (two Welsh Corgi, one Border Collie, one Beagle), two large-breed dogs (Bernese Mountain Dog), and thirteen cats, no noticeable correlation with body size was observed in this study. Furthermore, the AUC value was higher in cats than in dogs at lower sample concentrations. From the above, the optimum concentration of urine samples suitable for the N-NOSE test may be influenced not by the body size but by differences within animal species.

Our findings suggest that a subset of cancers in selected locations in both dogs and cats (liver, mammary gland, lymph node, thyroid, bladder, lung, kidney, and gastrointestinal tract) can be detected by the N-NOSE assay. For some cancer locations (jejunum, spleen, skin, nasal cavity) that have not been investigated for N-NOSE in humans, we did find distinct nematode reactions in our study for dogs or cats. Urothelial carcinoma, which is common in dogs, is very similar to human bladder cancer in terms of genetics, histopathology, and metastasis [36]. Interestingly, in this study, *C. elegans* nematodes showed attractive behavior towards the urine of dogs with urothelial carcinoma (dog 22). *C. elegans* were also attracted to the urine of a patient with a brain tumor (dog 21). The presence of the brain tumor in dog 21 was confirmed by MRI, but
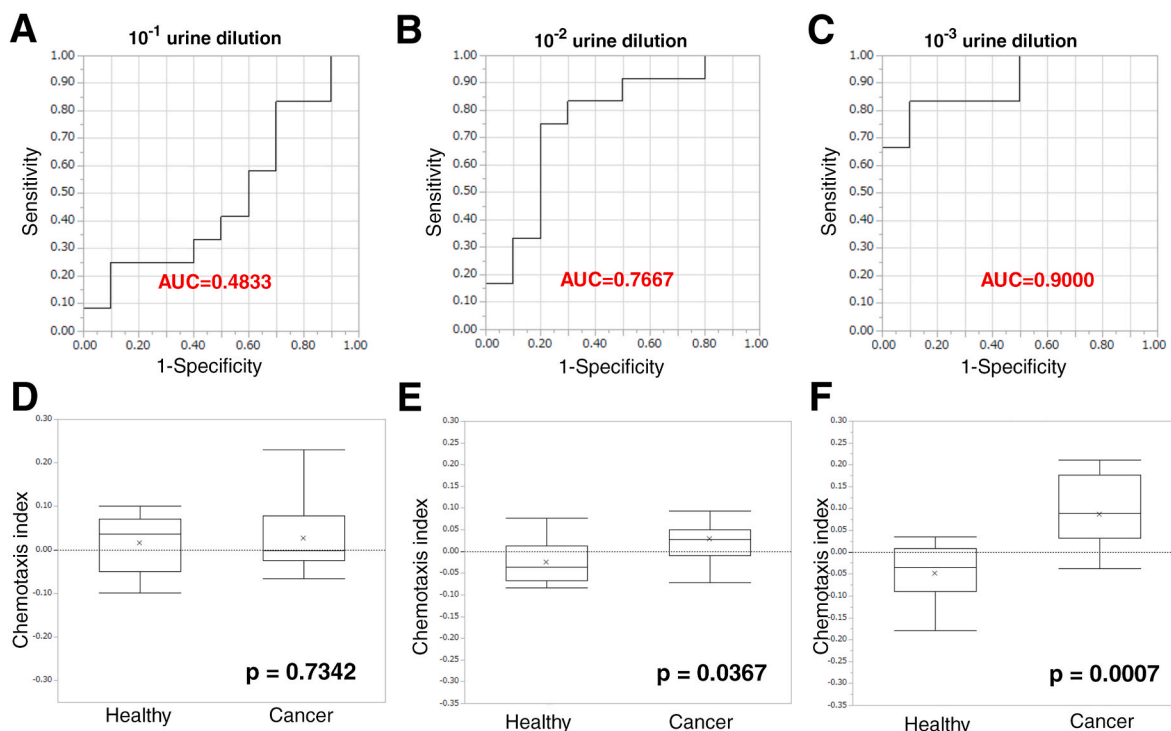
Fig. 2. N-NOSE assay results (malignant vs healthy) for diluted feline urine samples. **A–C**: Area under the curve (AUC) values at **A)** 10^{-1}, **B)** 10^{-2}, **C)** 10^{-3} dilution determined by receiver operating characteristic analysis. **D-F**: box plot of chemotaxis index of *C. elegans* to feline urine samples diluted at **D)** 10^{-1}, **E)** 10^{-2}, or **F)** 10^{-3} from 10 healthy cats and 13 cats with cancer. Error bars = SEM ($n \geq 3$ assays for all samples).

the benign or malignant nature of the tumor remains unknown, and it is also unclear how chemoattractant crossed the blood-brain barrier (BBB) and appeared in the urine. Molecules of <400 Da are documented to cross the BBB and the blood-CSF barrier, especially in the case of drug delivery [37].

Cancer treatment efficacy has been compared between canines and humans, showing notable similarities [38–40]. Based on comparative oncology findings across species, it is not unexpected that the N-NOSE assay can detect similar cancers in humans as well as in both felines and canines. N-NOSE for humans is a multi-cancer early detection test for regular screening. Our pilot study suggests that N-NOSE can potentially be used for regular cancer screening for cats and dogs, using urine samples, ideally as a substitute to invasive methods. Worth noting that in addition to showing a distinct chemotactic response to diluted urine samples between healthy and cancers, *C. elegans* also displays the same chemotaxis behavior with the supernatant of cancer cell cultures, but not for blood samples [20]. However, to determine the effectiveness of N-NOSE as a cancer screening test for pets, it may be necessary to consider further whether it can detect cancers that occur frequently in canines and felines. Although our data suggest that N-NOSE can detect the presence of cancers in both canines and felines, our study has several limitations. Mainly, the number of cases in our pilot study is small. In addition, given the inevitable constraints in subject recruitment, our study population comprised young non-tumor-bearing, and old tumor-bearing pets. Therefore, it will be necessary to investigate further in a larger and more detailed clinical study, especially considering the effect of canine and feline breeds. Following this pilot study, the next clinical study should consider the difference in reactivity among cancer types and the ability to separate benign and malignant tumors.

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Declaration of competing interest

T.S., N. K., T.H., and E.d.L. are employees of Hirotsu Bio Science Inc.

Data availability

Data will be made available on request.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2022.101332.

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