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Clinical experience with next-generation sequencing–based liquid biopsy testing for cancer detection in dogs: a review of 1,500 consecutive clinical cases

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OBJECTIVE
To review ordering patterns, positivity rates, and outcome data for a subset of consecutive samples submitted for a commercially available, blood-based multicancer early-detection liquid biopsy test for dogs using next-generation sequencing at 1 laboratory.

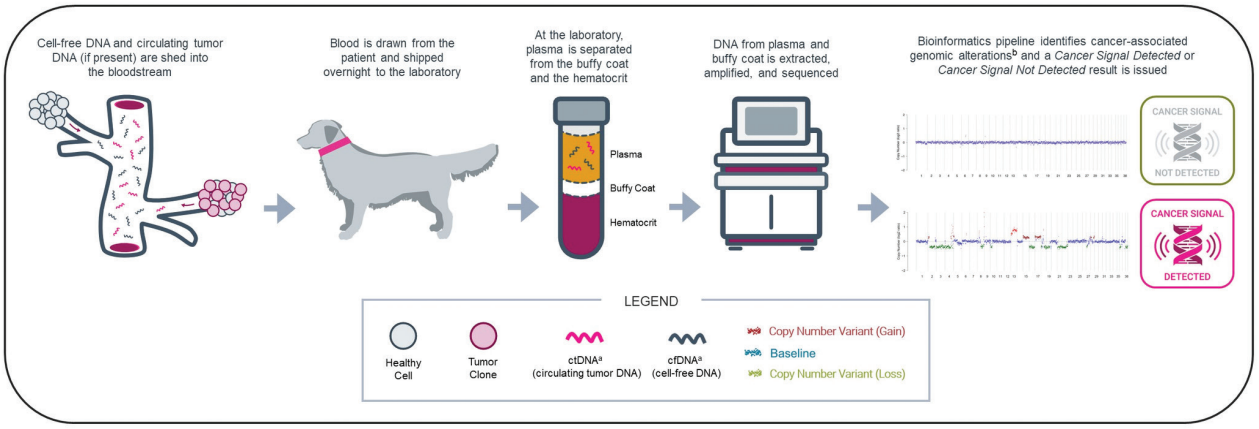
SAMPLE
1,500 consecutively submitted blood samples from client-owned dogs with and without clinical suspicion and/or history of cancer for prospective liquid biopsy testing between December 28, 2021, and June 28, 2022.

PROCEDURES
We performed a retrospective observational study, reviewing data from 1,500 consecutive clinical samples submitted for liquid biopsy testing. Outcome data were obtained via medical record review, direct communication with the referring clinic, and/or a patient outcome survey through October 16, 2022.

RESULTS
Sixty-four percent (910/1,419) of reportable samples were submitted for cancer screening, 26% (366/1,419) for aid in diagnosis, and 10% (143/1,419) for other indications. The positivity rate was 25.4% (93/366) in aid-in-diagnosis patients and 4.5% (41/910) in screening patients. Outcome data were available for 33% (465/1,401) of patients, and outcomes were classifiable for 428 patients. The relative observed sensitivity was 61.5% (67/109) and specificity was 97.5% (311/319). The positive predictive value was 75.0% (21/28) for screening patients and 97.7% (43/44) for aid-in-diagnosis patients, and the time to diagnostic resolution following a positive result was < 2 weeks in most cases.

CLINICAL RELEVANCE
Liquid biopsy using next-generation sequencing represents a novel tool for noninvasive detection of cancer in dogs. Real-world clinical performance meets or exceeds expectations established in the test's clinical validation study.

Blood-based liquid biopsy using next-generation sequencing involves the extraction and sequencing of cell-free DNA (cfDNA) from plasma in order to identify genomic alterations associated with cancer (eg, copy number gains/losses and DNA fragmentation profiles), as previously described.[1,2] Cell-free DNA comprises DNA shed from a variety of tissues throughout the body, including tumors (if present; **Figure 1**). Next-generation sequencing–based liquid biopsy for multicancer early-detection testing in dogs was validated in a study[3] of over 1,000 subjects and became clinically available in May 2021. Since



[a] Circulating tumor DNA (ctDNA) represents a subset of all cell-free DNA (cfDNA) fragments in circulation.
[b] Simplified graphic for illustrative purposes only. Multiple classes of genomic alterations may be associated with cancer, and not all are depicted.

Figure 1—Visual representation of the steps involved in next-generation sequencing–based liquid biopsy testing, from cell-free DNA entering the bloodstream through bioinformatics analysis at the laboratory.

that time, the liquid biopsy test has seen increasing utilization in various prediagnosis clinical scenarios, including as a cancer screening tool in dogs at higher risk for cancer based on age or breed and as an aid in diagnosis for dogs with clinical suspicion of cancer. Additional use cases for liquid biopsy include detection of residual disease following excisional surgery and detection of disease recurrence following therapy; evaluation of test performance in these postdiagnosis settings is an area of active research.

Once a test has been validated and is commercially available, it is important for the laboratory to periodically report utilization and performance metrics regarding the test. This study was designed to provide such information, including ordering patterns, positivity rates, and outcome data for samples submitted for commercial liquid biopsy testing at 1 clinical laboratory. The metrics described herein are derived from 1,500 consecutive samples received directly from veterinary clinics across the US and Canada.

Materials and Methods

Data analyzed in this study were from 1,500 consecutive clinical samples from 1,401 unique client-owned dogs received for liquid biopsy testing at 1 clinical laboratory (PetDx; La Jolla, CA). These samples were prospectively submitted by each patient's managing veterinarian as part of their recommended workup; the cancer status of the patient was not known to the referring veterinarian at the time of sample collection with the exception of 1 case (in which the owner was not convinced of a cancer diagnosis despite histopathology results). Testing was performed on whole blood samples (14 to 17 mL, collected from a jugular or peripheral vein) in collection tubes (Cell-Free DNA Collection Tubes; Roche). These tubes are stable at room temperature for 7 days, without refrigeration or freezing. Upon receipt at the laboratory, blood samples underwent a 2-step centrifugation process (15 minutes at 1,600 X *g*, then 10 minutes at 2,500 X *g*) to separate plasma from WBCs.[2,3] Cell-free DNA was extracted from plasma using a proprietary bead-based chemistry optimized to maximize cfDNA yield in canine subjects. Genomic DNA was extracted from WBCs via a kit (QIAamp DNA Mini Blood Kit; Qiagen). Amplified DNA libraries were generated for each subject from the matched cfDNA and genomic DNA extracts. Libraries were prepared by incorporating universal adapters and barcodes into sample DNA via ligation and universal PCR amplification and were subjected to next-generation sequencing on a genome-sequencing system (NovaSeq 6000; Illumina Inc) for somatic variant analysis. All sequencing reads were aligned to the CanFam3.1 reference genome,[4] and variant calls were made via custom bioinformatics pipelines as previously described.[3] Test results were issued by the laboratory team without a priori knowledge of patient signalment, clinical signs, or other case details.

The indication for testing for each sample was assigned based on the information provided by the referring veterinarian on the electronic test requisition form and review of any additional patient information as provided by the referring clinic. For cases in which there was no current suspicion of cancer, the indication for testing was classified as "screening"; for cases in which there was a current suspicion of cancer, the indication for testing was classified as "aid in diagnosis"; for cases in which a patient received recent treatment (surgical or nonsurgical) for cancer, the indication for testing was classified as "postdiagnosis"; for cases in which no information was provided, the indication for testing was classified as "not provided."

Upon release of the test results from the laboratory, a report was issued to the referring veterinarian using a standard workflow. Results were classified into 3 categories: *Cancer Signal Detected* (positive), *Cancer Signal Not Detected* (negative), or *Indeterminate* (genomic alterations were detected, but their significance was uncertain; in these cases, a complimentary retest was offered). A subset of patients receiving a *Cancer Signal Detected* result had genomic

alterations consistent with hematologic malignancy as previously described[3] and were also provided a corresponding cancer signal origin (CSO) prediction. Additional cancer types are expected to be available for CSO prediction in the future as further cancer-specific signatures are identified and validated. Samples that were received or processed more than 7 days from the time of collection, samples in which blood tubes did not meet minimum fill requirements, and samples that did not pass quality control metrics were categorized as *Failures*, and a complimentary retest was offered.

Outcome data were obtained from referring veterinary clinics via email and/or phone. The clinics were provided with a list of patients for which liquid biopsy testing was ordered from their site, and they were given the option to complete a patient-outcomes survey **(Supplementary Figure S1)** and/or to attach recent medical records; for each patient that had outcome data provided, the clinic was issued a $10 gift card. The survey was a web-based form that was accessible for 33 days (from September 13, 2022, through October 16, 2022) and consisted of questions including the following: Has the patient been diagnosed with cancer? What was the type and anatomic location(s) of the cancer? Was the diagnosis definitive (tissue-based; ie, via cytology or histopathology) or presumptive (based on imaging, direct examination/visualization, or strong suspicion on cytology/histopathology)? When was the clinic's last contact with the pet owner for a patient status update? If the patient was deceased, the following question was also included: What was the date of death, and did the patient die due to suspected or known cancer? For clinics that elected to send medical records in lieu of completing the survey or in conjunction with the survey, the records were reviewed by DVM coauthors including American College of Veterinary Internal Medicine board-certified specialists in small animal internal medicine and oncology; this review included a verification of all records for case outcome classification. In a subset of additional cases (n = 60), patient outcomes had already been documented from historic conversations, emails, or medical records from the referring veterinarians. In those cases, the outcome data were entered by a DVM coauthor into an internal version of the patient-outcomes survey and also reviewed by American College of Veterinary Internal Medicine board-certified specialist coauthors.

Cases were classified into outcome categories based on the patient's liquid biopsy result and the follow-up information available as of the date the study outcome collection closed. For patients with *Cancer Signal Not Detected* (negative) liquid biopsy results, cases were classified as true negative (TN) or false negative (FN). TN was used for cases in which the clinic indicated "Cancer has not been diagnosed, nor is cancer suspected" and there was no ongoing cancer workup in progress, and for cases where no evidence of a cancer diagnosis was noted from review of the patient's medical record. The classification of TN was made based on workup in cases that had cancer evaluations. The components of individual workups were variable but typically included a physical examination, with or without lab work, imaging, and sampling of suspicious nodules/masses. Cancer evaluations were not performed in all patients, nor was it possible to completely rule out the presence of cancer in each patient. In such cases, the classification of TN was assumed based on available data at the time the study outcome collection closed, including but not limited to veterinarian knowledge of the patient's status via contact with the owner and recent examination results. FN was used for cases in which cancer was diagnosed at any point following liquid biopsy testing. For patients with an initial test failure or *Indeterminate* result, outcome classifications were assigned based on the patient's final results from repeat testing (if performed).

For patients with *Cancer Signal Detected* (positive) liquid biopsy results, cases were classified as true positive (TP) or false positive (FP); the TP category included subclassifications of TP—definitive diagnosis or TP—presumptive diagnosis. Cases classified as having a definitive diagnosis were cases in which tissue-based cancer diagnosis was made via cytology/histopathology; those classified as having a presumptive diagnosis were based on imaging, direct visualization/examination, or strong suspicion of cancer from cytology or histopathology. Cases classified as FP received complete workup as of the date the study outcome collection closed, with no cancer diagnosis made; where applicable, these cases continue to be monitored by the referring veterinarian.

A subset of *Cancer Signal Detected* and *Cancer Signal Not Detected* cases could not be classified into an outcome category based on the available information at the time the study outcome collection closed. This included cases where a cancer evaluation was incomplete or in progress or the dog was lost to follow-up or euthanized (due to clinical presentation or quality of life) with limited or no workup.

The demographic data were summarized by patient rather than by sample, meaning that for patients with more than 1 sample submitted for testing during the study period, analysis was based on demographic information provided for the patient's first submitted sample. Analyses of test indication, positivity rate, and turnaround time were performed at the individual sample level. Positivity rate = [positive results/(positive + negative + indeterminate results)] X 100.

An assessment of observed test performance metrics (sensitivity, specificity, positive predictive value [PPV], and negative predictive value [NPV]) was made in the population of patients assigned an outcome classification, as defined above. Relative observed sensitivity was defined as the rate of liquid biopsy *Cancer Signal Detected* results in patients with definitive or presumptive cancer diagnoses, and relative observed specificity was defined as the rate of *Cancer Signal Not Detected* results in patients in which cancer was not diagnosed or suspected. Relative observed PPV was defined as the rate of cancer diagnoses (definitive + presumptive) in patients that received *Cancer Signal Detected* results, and relative observed NPV was defined as the rate of noncancer in patients that received

Cancer Signal Not Detected results. For statistical analyses, calculation of *P* values was performed using a 2-sided *t* test in the case of continuous variables and a χ^2 test in the case of categorical variables; $P < .05$ was considered statistically significant.

Results

Patient characteristics

The 1,500 samples analyzed for this study originated from 1,401 unique patients from across the US and Canada. The patients ranged in age from < 1 to 18 years, with a median age of 9.3; weight data were available for 1,283 patients, and weights ranged from 1.8 to 82.0 kg, with a median of 26.3 kg; sex was provided for over 99% (1,389/1,401) of patients, of which approximately 52% (725/1,401) were male and 47% were female (664/1,401); spay-neuter status was documented for over 98% (1,376/1,401) of patients, of which 93% were spayed or neutered (1,278/1,376); 61% (849/1,401) of patients were reported as purebred and 39% (552/1,401) as mixed breed **(Table 1)**.

Overview of results

For the 1,500 consecutive samples analyzed, 5.3% (80/1,500) failed quality control metrics, and in 1 case the client requested to withdraw testing, leaving 1,419 reportable samples, which comprised 156 *Cancer Signal Detected*, 1,222 *Cancer Signal Not Detected*, and 41 *Indeterminate* samples. Of the 156 positive results issued, 13% (20/156) received a CSO prediction of hematologic malignancy.

Of the 80 samples that failed quality control metrics, 62 samples failed due to insufficient blood sample volume and 19 failed for other reasons, including transit time or sample issue during transit (n = 9), sequencing-related or laboratory processing issue (8), and expired tubes (1).

Indications for testing

Of the 1,419 reportable samples in this study, 64% (910/1,419) were submitted for liquid biopsy as a screening test (the veterinarian did not clinically suspect cancer, but deemed the patient to be at higher risk for cancer based on age or breed); 26% (366/1,419) were submitted as an aid-in-diagnosis test (the veterinarian had a clinical suspicion of cancer); 3% (48/1,419) were submitted outside the intended use of the test, for a postdiagnosis indication (including residual disease detection, recurrence monitoring, or treatment response monitoring); and 7% (95/1,419) were submitted without a specified indication for testing.

Positivity rates by test indication

The highest test positivity rate (25.4%; 93/366) was seen in the aid-in-diagnosis population, and the lowest positivity rate (4.5%; 41/910) was seen in the screening population **(Table 2)**.

Table 1—Demographic characteristics of the 1,401 patients that had samples submitted for blood-based multicancer early-detection liquid biopsy testing between December 28, 2021, and June 28, 2022, with comparisons of demographic characteristics of patients that received *Cancer Signal Not Detected* (*CSND*; n = 1,144) and *Cancer Signal Detected* (*CSD*; 140) results. An additional 117 patients received *Indeterminate* results or experienced a test failure and were not included in the comparison.

Demographic characteristics	All patients (n = 1,401)	Patients with *CSND* results (n = 1,144)	Patients with *CSD* results (n = 140)	*CSND*:*CSD P* value
Age				*P* = .002
No. of patients	1,401	1,144	140	
Range (y)	< 1–18	< 1–18	1.5–17	
Median (y)	9.3	9.1	10.0	
Mean (y)	9.2 ± 3.1	9.1 ± 3.1	9.9 ± 2.9	
Weight				*P* = .675
No. of patients	1,283[a]	1,059[b]	121[b]	
Range (kg)	1.8–82	1.8–82.0	3.2–65.8	
Median (kg)	26.3	26.8	27.9	
Mean (kg)	25.2 ± 13.8	25.5 ± 13.7	26.0 ± 13.5	
Sex				*P* = .502 (male to female)
Male	725 (52%)	593 (52%)	69 (49%)	
Neutered	657	536	62	
Intact	63	55	5	
Neuter status not provided	5	2	2	
Female	664 (47%)	541 (48%)	71 (51%)	
Spayed	621	509	61	
Intact	35	26	9	
Spay status not provided	8	6	1	
Sex not provided	12 (< 1%)	10	0	
Breed				*P* = .353
Purebred	849 (61%)	689 (60%)	90 (64%)	
Mixed breed	552 (39%)	455 (40%)	50 (36%)	

Calculation of *P* values was performed using a 2-sided *t* test in the case of continuous variables (ie, age and weight), and a χ^2 test in the case of categorical variables (ie, sex and breed); $P < .05$ was considered statistically significant.

[a]Weight was not provided for 118 patients in the overall cohort. [b]Weight was not provided for 85 patients with CSND results and 19 patients with CSD results.

Table 2—Distribution of liquid biopsy results (ie, *CSD* [positive], *CSND* [negative], and *Indeterminate*) and positivity rates for the 1,419 reportable samples originating from a subset of the 1,401 patients described in Table 1, stratified by the indicated purpose for testing: screening, aid in diagnosis, postdiagnosis, and not provided.

Indication	Overall	Screening	Aid in diagnosis	Postdiagnosis	Not provided
Number of reportable samples	1,419	910	366	48	95
CSD (positive)	156	41	93	8	14
CSO: hematologic malignancy	20 of 156	6 of 41	11 of 93	0 of 8	3 of 14
CSND (negative)	1,222	844	260	39	79
Indeterminate	41	25	13	1	2
Positivity rate[a]	11.0%	4.5%	25.4%	16.7%	14.7%

CSO = Cancer signal origin.
Reportable samples are those in which a *CSD*, *CSND*, or *Indeterminate* result was issued (excludes test failures).
[a]Positivity rate = [positive results/(positive + negative + indeterminate results)] X 100.

The demographic characteristics (ie, age, weight, ratio of males to females, ratio of purebred to mixed-breed dogs) of the patients that received *Cancer Signal Not Detected* results were compared with those of the patients that received *Cancer Signal Detected* results. The only significant difference was that dogs with *Cancer Signal Detected* results were, on average, older than the dogs with *Cancer Signal Not Detected* results (mean, 9.9 ± 2.9 years vs 9.1 ± 3.1 years; $P = .002$; Table 1).

Turnaround time

The median turnaround time for the 1,419 reportable samples was 11 (IQR, 8 to 13) calendar days. When comparing the first half of samples submitted for testing (n = 710) to the second half (709), there was a significant decrease in average turnaround time, from 11.2 to 10.2 calendar days ($P < .0001$).

Patients with failed or indeterminate results

Of the 81 patients that had an initial failed test, 55 elected to submit a second sample (redraw); 96% of these patients (53/55) received a clear positive or negative result from the redraw (2 positives and 51 negatives); 1 returned an *Indeterminate* result on the first redraw followed by a negative result on second redraw; and 1 returned an additional test failure on the first redraw followed by a negative result on second redraw.

Of the 41 patients that had an initial *Indeterminate* result, 18 had a second sample submitted for testing; 89% of these patients (16/18) received a clear positive or negative result from the redraw. Results of repeat testing included 11 negative results (including 1 patient that had 2 repeat negative tests), 5 positive results, and 2 repeat *Indeterminate* results with no further testing.

Outcome data: overall study population

Of the 1,401 patients in the study population, clinical outcome data were received for 33% (465/1,401). For 174 patients, medical records were available for review; for 60 patients, outcome information was documented from historic conversations or emails from the referring veterinarian; and for 231 patients, a patient-outcomes survey was completed (for 34 of these 231 patients, medical records were provided in addition to completion of the survey).

Of the 465 patients with outcome data available, it was possible to assign an outcome classification, as previously described, in 428 cases **(Table 3)**.

Table 3—Liquid biopsy test performance metrics based on available clinical outcome data for 428 of the 1,401 patients described in Table 1 with samples submitted for any test indication (ie, screening, aid in diagnosis, postdiagnosis, and not provided). An additional 37 cases (20 negative, 9 positive, 5 indeterminate, 3 failure) had outcome data provided but could not be assigned to the 2 X 2 table. For the cases with positive or negative results, these included situations where confirmatory cancer evaluations were incomplete or in progress and cases that were lost to follow-up (or euthanized) with limited or no workup performed.

	Cancer status		
Liquid biopsy result	**Present (n = 109)**	**Absent (n = 319)**	
CSD (n = 75)	TP,[a] 67	FP, 8	Relative observed PPV, 89.3% (95% CI, 79.5%–95.0%)
CSND (n = 353)	FN,[b] 42	TN,[c] 311	Relative observed NPV, 88.1% (95% CI, 84.2%–91.2%)
	Relative observed sensitivity,[d] 61.5% (95% CI, 51.6%–70.5%)	Relative observed specificity,[e] 97.5% (95% CI, 94.9%–98.8%)	

FN = False negative. FP = False positive. NPV = Negative predictive value. PPV = Positive predictive value. TN = True negative. TP = True positive.
[a]TP = 39 presumptive, 28 definitive. [b]FN = 16 presumptive, 26 definitive. [c]Assumed TNs based on available data at the time study outcome collection closed. [d]The sensitivity observed in the clinical validation study for the test (CANcer Detection in Dogs [CANDiD] study) was 54.7% (95% CI, 49.3% to 60.0%).[3] [e]The specificity observed in the clinical validation study for the test (CANDiD study) was 98.5% (95% CI, 97.0% to 99.3%).[3]
Relative observed sensitivity = TP/[TP + FN]. Relative observed specificity = TN/[TN + FP]. Relative observed PPV = TP/[TP + FP]. Relative observed NPV = TN/[TN + FN].

The relative observed sensitivity in the population of patients with an outcome classification was 61.5% (67/109; 95% CI, 51.6% to 70.5%), and relative observed specificity was 97.5% (311/319; 95% CI, 94.9% to 98.8%). These performance metrics were within or above the confidence intervals for expected test performance as established in the CANcer Detection in Dogs (CANDiD) clinical validation study.[3] Specifically, in the current study the relative observed specificity of the test (97.5%) was within the 95% CI of specificity from the CANDiD study (97.0% to 99.3%), and the relative observed sensitivity of the test (61.5%) was higher than the upper bound of the 95% CI of sensitivity from the CANDiD study (49.3% to 60.0%).[3]

A wide range of cancer types were represented in the cancer-diagnosed patients with outcome classifications **(Supplementary Table S1)**. For these patient, definitive diagnoses were listed by histologic type, and presumptive diagnoses were listed by anatomic location. The most common definitive diagnosis was lymphoma/acute lymphoid leukemia (11/54 [20%]), and the most common presumptive diagnoses involved the spleen (6/55 [11%]) and the liver (5/55 [9%]).

Of the 428 patients with outcome classifications, 8 were classified as FPs: 7 were submitted as screening tests, and 1 was submitted as an aid-in-diagnosis test. All 8 patients saw a specialist (including internists, radiologists, and/or oncologists) and had workups that included detailed history, physical examination, routine labs (including CBC, biochemical analyses, and urinalysis), thoracic radiography, and abdominal ultrasonography; some patients also had lymph node or organ fine-needle aspirate (FNA; 2 patients), echocardiogram (1 patient), and urine *BRAF* testing for transitional cell carcinoma/urothelial carcinoma[5] (1 patient). At the time the study outcome collection closed, 7 of the 8 patients continued to be followed in a company-sponsored monitoring program; 4 of these 7 patients had findings that could be consistent with early cancer, including prominent sublumbar lymph nodes, collapse/seizure-like events, splenic nodules on ultrasonography, and subtle gastrointestinal thickening, respectively. Therefore, while these cases were classified as FPs for the purpose of this study, there is potential for future adjudication as workup continues in these patients. There was no observed enrichment in FPs as a function of either age or sex (4 patients were at or below the median age of the study population, and 4 were above; 5 patients were male, and 3 were female); however, as expected, patients with confirmed cancer tended to be older than patients without cancer (62.4% of patients with cancer vs 45.1% of patients without cancer were above the median age of the study population; Fisher exact $P = .002$).

There were 42 patients with outcomes classified as FNs: 15 were submitted as screening tests, and 27 were submitted as aid-in-diagnosis tests. For the screening patients, the date of cancer diagnosis was provided for 10 of the 15 patients, and the median time from receipt of the negative test result to the diagnosis of cancer was 68 days (range, 0 to 195 days); 1 dog received a diagnosis between the date of the blood draw and the date of the liquid biopsy report and was assigned a time to diagnosis of 0 days. The FN cases comprised mast cell tumor (3 cases, all diagnosed 1 to 3 weeks after liquid biopsy), masses in the liver or spleen (3 cases, diagnosed 1 week to 6 months after liquid biopsy), parathyroid tumor (1 case, diagnosed 5 months after liquid biopsy), mammary gland tumor (2 cases, both diagnosed 5 months after liquid biopsy), and intermediate B-cell lymphoma (1 case, diagnosed 5 months after liquid biopsy). Five additional screening patients with the following cancer types were diagnosed without a date of cancer diagnosis provided: liver, adrenal gland, trunk/extremities (soft tissue sarcoma), lung + heart (suspected hemangiosarcoma), and anal sac + heart base.

For 289 of the 311 patients with TN results, the clinic provided the date that they last had contact with the owner for a patient status update. The median time that had elapsed from the patient's liquid biopsy result to the date of last owner contact was 119 days (range, 0 to 274 days). From the date the study outcome collection closed, 96% (276/289) of clinics had contact with the owner within the previous 6 months, 77% (222/289) had contact within the previous 3 months, and 30% (88/289) had contact within the previous 1 month (median, 44 days; range, 6 to 282 days).

It should be noted that there were 13 cases in which the survey completed by the veterinary clinic staff indicated that cancer was definitively or presumptively diagnosed but the case outcome was ultimately classified as TN based on medical record review or follow-up conversations with the clinic. These 13 patients are included in the 311 cases classified as TN. Nine of these cases had samples submitted for postdiagnosis indications (including residual disease detection, recurrence monitoring, and treatment response monitoring), and all cases were negative by liquid biopsy with no evidence of disease on clinical examination. In 2 cases, the dog was diagnosed with cancer 1 to 2 years prior and had achieved complete remission, and the families were pursing liquid biopsy as a screening test to look for new cancer (or, potentially, recurrence of the previous cancer). In 1 case, the survey indicated a presumptive diagnosis, but additional testing (cytology) performed after the survey response was submitted was negative for malignancy. In the final case, the clinic misread medical records and incorrectly attributed a diagnosis of cancer in the family's previous dog to the otherwise healthy dog being screened. All other cases in which both a survey was completed and medical records were available for review were concordant in outcome classifications.

Of the 67 cases designated as TPs, a date of cancer diagnosis was provided for 41 patients that submitted testing for screening or as an aid in diagnosis. Using this information, along with the date of the patient's positive liquid biopsy result, a time to diagnostic resolution (ie, time elapsed from receipt of the positive liquid biopsy result to confirmation of cancer diagnosis) was calculated. The median time to diagnostic resolution was 11 days, with a range from 0 to 56 days. When these data were analyzed by test indication, the median time to diagnostic resolution

was 11 days (n = 13; range, 0 to 44 days) for screening patients and 12 days for aid-in-diagnosis patients (28; range, 0 to 56 days; **Supplementary Figure S2**).

Outcome data: screening patients

Clinical outcome data were received for 299 patients with samples submitted for liquid biopsy as a screening test, of which it was possible to assign an outcome classification in 286 patients **(Table 4)**. The relative observed PPV in the screening population was 75.0% (21/28; 95% CI, 54.8% to 88.6%), meaning that three-quarters of screening patients that received a positive result from liquid biopsy testing and went on to have a confirmatory cancer evaluation received a definitive or presumptive diagnosis of cancer. The relative observed NPV in the screening population was 94.2% (243/258; 95% CI, 90.4% to 96.6%), meaning that the vast majority of patients that received a negative result from liquid biopsy testing were not diagnosed with or suspected to have cancer at the time the study outcome collection closed.

In the screening cohort, there were 21 patients with outcomes classified as TP. The workups that were performed for these cases were known in 17 of 21 patients; 15 patients had abdominal imaging (13 underwent abdominal ultrasonography and 2 underwent abdominal radiography), 12 had thoracic imaging (thoracic radiography alone, n = 10; thoracic ultrasonography alone, 1; or thoracic radiography and ultrasonography, 1), 9 had FNA cytology (6 were ultrasound guided), and 2 had histopathology; 2 had additional testing unique to the case, including urine *BRAF* testing in 1 dog and lymph node PCR for antigen receptor rearrangement and flow cytometry in 1 dog. In 15 of the 17 patients, the diagnosis was achieved through a workup performed within the practice of the veterinarian that had ordered the liquid biopsy test; in the other 2 cases, the patient was sent to a specialist for the workup (an oncologist in both cases), which established the cancer diagnosis.

Outcome data: aid-in-diagnosis patients

Clinical outcome data were received for 151 patients with samples submitted for liquid biopsy as an aid-in-diagnosis test, of which it was possible to assign an outcome classification in 127 patients **(Table 5)**.

Table 4—Liquid biopsy test performance metrics for a subset of 286 patients with clinical outcome data that had samples submitted for cancer screening. These 286 patients represent a subset of the 428 patients described in Table 3. An additional 13 cases (6 negative, 3 positive, 2 indeterminate, 2 failure) had outcome data provided but could not be assigned to the 2 X 2 table. For the cases with positive or negative results, these included situations where confirmatory cancer evaluations were incomplete or in progress and cases that were lost to follow-up (or euthanized) with limited or no workup performed.

	Cancer status		
Liquid biopsy result	**Present (n = 36)**	**Absent (n = 250)**	
CSD (n = 28)	TP,[a] 21	FP, 7	Relative observed PPV,[d] 75.0% (95% CI, 54.8%–88.6%)
CSND (n = 258)	FN,[b] 15	TN,[c] 243	Relative observed NPV,[e] 94.2% (95% CI, 90.4%–96.6%)
	Relative observed sensitivity, 58.3% (95% CI, 40.9%–74.0%)	Relative observed specificity, 97.2% (95% CI, 94.1%–98.8%)	

[a]TP = 12 presumptive, 9 definitive. [b]FN = 7 presumptive, 8 definitive. [c]Assumed TNs based on available data at the time study outcome collection closed. [d]The PPV estimate made in the clinical validation study for the test (CANDiD study) was 76% to 80%.[3] [e]The NPV estimate made in the clinical validation study for the test (CANDiD study) was 95% to 96%.[3]

See Table 3 for remainder of key.

Table 5—Liquid biopsy test performance metrics for a subset of 127 patients with clinical outcome data that had samples submitted as an aid in diagnosis. These 127 patients represent a subset of the 428 patients described in Table 3. An additional 24 cases (14 negative, 6 positive, 3 indeterminate, 1 failure) had outcome data provided but could not be assigned to the 2 X 2 table. For the cases with positive or negative results, these included situations where confirmatory cancer evaluations were incomplete or in progress and cases that were lost to follow-up (or euthanized) with limited or no workup performed.

	Cancer status		
Liquid biopsy result	**Present (n = 70)**	**Absent (n = 57)**	
CSD (n = 44)	TP,[a] 43	FP, 1	Relative observed PPV,[d] 97.7% (95% CI, 86.5%–99.9%)
CSND (n = 83)	FN,[b] 27	TN,[c] 56	Relative observed NPV,[e] 67.5% (95% CI, 56.2%–77.1%)
	Relative observed sensitivity, 61.4% (95% CI, 49.0%–72.6%)	Relative observed specificity, 98.2% (95% CI, 89.4%–99.9%)	

[a]TP = 25 presumptive, 18 definitive. [b]FN = 9 presumptive, 18 definitive. [c]Assumed TNs based on available data at the time study outcome collection closed. [d]The PPV estimate made in the clinical validation study for the test (CANDiD study) was 94% to 97%.[3] [e]The NPV estimate made in the clinical validation study for the test (CANDiD study) was 68% to 84%.[3]

See Table 3 for remainder of key.

The relative observed PPV in the aid-in-diagnosis population was 97.7% (43/44; 95% CI, 86.5% to 99.9%), meaning that nearly 98% of patients that received a positive result from liquid biopsy testing went on to receive a definitive or presumptive diagnosis of cancer. The relative observed NPV in the aid-in-diagnosis population was 67.5% (56/83; 95% CI, 56.2% to 77.1%), meaning that most patients that received a negative result from liquid biopsy testing were not diagnosed with (and were not suspected to have) cancer at the time the study outcome collection closed; however, cancer was definitively or presumptively diagnosed in approximately 32% of patients that had received a negative liquid biopsy result.

The reason for submitting a liquid biopsy sample for aid-in-diagnosis testing was available for 124 patients. The majority of patients (59% [73/124]) had samples submitted due to clinical suspicion of cancer based on presenting history, results of physical examination, and/or lab results; 32 patients (26% [32/124]) had samples submitted due to suspicion of cancer from imaging; and 14 patients (11% [14/124]) had samples submitted due to inconclusive tissue testing (2 of which had inconclusive cytology followed by loss of histopathology samples in transit to the diagnostic pathology laboratory). Additional reasons for using the test as an aid in diagnosis included failure to achieve a diagnosis despite extensive workup (n = 2), helping to convince the owner of the cancer diagnosis (2), and owner suspicion of cancer (1).

Outcome data: patients that received a CSO prediction

In the overall cohort of 1,500 samples, 20 positive results were issued with a CSO prediction indicating the likely presence of a hematologic malignancy (lymphoma or lymphoid leukemia). Of these 20 cases, outcome classifications were available for 13 patients: 11 received a cancer diagnosis (TPs), and 2 did not receive a cancer diagnosis following a full cancer evaluation (FPs). Information was provided for 1 additional dog; however, the confirmatory cancer evaluation was ongoing, and the patient could not yet be assigned an outcome classification. Nine out of 13 (69%) patients that received CSO predictions of hematologic malignancy and had classifiable outcomes were diagnosed with (or were suspected to have) lymphoma, and 2 additional patients had presumptive cancer but lymphoma was not the primary differential diagnosis (although it could not be ruled out). The 9 patients with definitive or presumptive lymphoma included 6 patients diagnosed with lymphoma/acute lymphoid leukemia, 1 with indolent lymphoma, 1 patient with presumptive lymphoma based on hypercalcemia as well as splenic and hepatic nodules with an appearance typical of lymphoma, and 1 patient with a caudal abdominal mass or lymph node in the pelvic canal with a hypoechoic appearance on ultrasonography. The 2 patients with presumptive cancer in which lymphoma was not the primary differential diagnosis had pancreatic as well as submucosal bladder masses (1 patient) and a suspected nasal mass (1 patient).

Adverse events

There were no reported adverse events as a direct result of liquid biopsy testing (ie, from venipuncture). There were also no reported adverse events due to diagnostic workups prompted by *Cancer Signal Detected* results in screening patients. Two patients with suspected cancer (submitted as aid-in-diagnosis samples) had adverse events during their clinical workup: one of those patients had signs of acute abdominal pain 1 week after ultrasound-guided FNA of a pancreatic mass and bruising on the ventral abdomen at the site of the aspirate, and the other had pathologic fracture of the tibia 2 weeks after biopsy of an aggressive bone lesion.

Discussion

This study of 1,500 consecutive samples submitted to 1 central lab for liquid biopsy testing demonstrated that the majority of samples were submitted for cancer screening, followed by aid-in-diagnosis testing; as expected, the positivity rate of liquid biopsy varied with the indication for testing. Outcome data were available for one-third of patients in the study; in patients that received a positive (*Cancer Signal Detected*) result, cancer was confirmed in nearly 90% of dogs, with most patients achieving diagnostic resolution within 2 weeks of receiving the result.

The patients in this study represented the range of ages, weights, and breeds seen in routine clinical practice. Nearly two-thirds of samples were submitted for cancer screening in dogs for which there was no clinical suspicion of cancer; the positivity rate of liquid biopsy testing was lowest, as expected, in this population, at 4.5%. Approximately one-quarter of samples were submitted for aid-in-diagnosis testing in dogs suspected of having cancer; the positivity rate was higher, as expected, in this population, at 25.4%.

Of the 428 patients with classifiable outcomes, 109 had received a definitive or presumptive diagnosis of cancer, and 319 had no diagnosis of cancer as of the time the study outcome collection closed. The relative observed sensitivity and specificity of the liquid biopsy test (61.5% and 97.5%, respectively) were within or above the confidence intervals for performance established in the test's clinical validation (CANDiD) study (49.3% to 60.0% and 97.0% to 99.3%, respectively).[3] This suggests that the use of independent training and testing sets in the CANDiD study may have mitigated (as intended) the possibility of data overfitting, allowing for reliable generalizability of the test's clinical performance metrics to a real-world clinical population.[6,7]

The relative observed PPVs (ie, the percentage of dogs with a *Cancer Signal Detected* result that were subsequently diagnosed with cancer) in screening and aid-in-diagnosis patients (75.0% and 97.7%, respectively) were also similar to the PPV estimates made in the CANDiD study (76% to 80% and 94% to 97%, respectively). This similarity suggests that the estimates for cancer prevalence used in the CANDiD study (ie, 8% to 10% in screening patients from high-risk groups and 30% to 50% in aid-in-diagnosis

patients) closely approximate the true prevalence of cancer in these populations. Importantly, the 75% screening PPV for multicancer detection by next-generation sequencing–based liquid biopsy in dogs compares favorably with PPVs observed in cancer screening programs for human patients, such as ≤ 30% for mammography (breast cancer screening), for a multitarget stool DNA test (colorectal cancer screening), and for prostate-specific antigen screening.[8–10] Furthermore, a recent prospective study[11] of a blood-based multicancer early-detection test for human patients documented a PPV of 38%.

The high specificity and PPV of next-generation sequencing–based liquid biopsy testing mean that FPs are rare. This is of particular importance in the screening setting, when testing large populations of dogs with no current suspicion of cancer. An FP result in a screening patient could lead to unnecessary invasive tests as well as anxiety and financial expense to the owner.

When cancer was detected by the liquid biopsy test, most patients who went on to receive a definitive or presumptive diagnosis of cancer achieved diagnostic resolution within 2 weeks, and all had received a diagnosis within 2 months. Therefore, the "diagnostic odyssey" for patients with positive liquid biopsy results appears to be relatively short, even in patients that were referred for screening and had no prior suspicion of cancer. Furthermore, for screening patients that had positive liquid biopsy results and went on to receive a cancer diagnosis, the diagnostic workup was typically performed in-house at the referring veterinary clinic and rarely required referral to a specialist.

A variety of cancer types were detected by liquid biopsy testing. Of particular interest were cancer types detected by the test in screening patients that were not clinically detected at that patient's blood draw appointment. These represented cancer types that may be challenging to detect on a wellness examination of patients not showing related clinical signs and included acute lymphoid leukemia (with splenic involvement), brain tumor, hemangiosarcoma (cardiac and splenic), histiocytic sarcoma (pulmonary), lymphoma (splenic), and cancers of the abdominal cavity, liver, lungs, intestines, and spleen. This suggests that the addition of liquid biopsy to a routine wellness visit may help to expand the number of cancer cases, and the range of cancer types, that can be detected preclinically (when patients are still not showing clinical signs).

For cases classified as TN, the average amount of time that had elapsed from the patient's negative liquid biopsy result to the time the clinic last had contact with the owner for a patient status update was approximately 4 months. Furthermore, the median time that had elapsed from last owner contact to the time the study outcome collection closed was 44 days. Therefore, most cases classified as TN had recent information available to support this outcome classification.

The strength of this observational study was the large cohort of patients studied and the availability of outcome data on a substantial number of patients (465 [33%]). However, there were also limitations to note.

The primary limitation lies in the collection of outcome data. For clinics that elected to answer survey questions about clinical outcomes without providing corresponding medical records, it was not possible for the study coauthors to confirm the accuracy of the information submitted. Additionally, in some cases, dogs may have received care at more than 1 veterinary hospital, so the provided medical records and outcomes may have been incomplete for some patients.

Another limitation of this study was that some patients did not have a thorough physical examination (including palpation of lymph nodes, oral and rectal examinations, etc) at the time blood was drawn for liquid biopsy testing; therefore, clinical status at the time of testing could not be confirmed for every case. Clinical status is important when considering the use case for each particular test, so it is possible that some cases that were submitted as screening tests would have actually been categorized as aid-in-diagnosis tests, had clinical status been assessed more closely at the time of the blood collection.

Additionally, cases were classified as TN based on medical record review or completion of the patient-outcomes survey, but not all dogs received a full workup to ensure the absence of cancer. This limitation, which reflects the real-world nature of this observational study, means that some of the cases categorized as TN may actually have had undiagnosed cancer during the study period.

Cases with positive results and presumptive diagnoses from the managing veterinarian (ie, those made without histopathologic or cytologic confirmation) could not be fully adjudicated as malignant rather than benign. Though prior work[3] suggests that benign masses are unlikely to produce a *Cancer Signal Detected* result, the possibility cannot be ruled out in this study.

Lastly, as with any outcome data collection that relies on voluntary response, a self-selection bias was possible; clinics may have been more or less likely to provide outcome data based on the type of data they were submitting. For instance, some clinics may be more likely to report outcome data in cases where results were discordant with the patient's outcome (eg, FNs and FPs), while other clinics may be less likely to report data if they have had discordant outcomes. It is unclear how these unknown factors might have biased test performance calculations that relied upon the outcome data collection.

In conclusion, blood-based liquid biopsy offers clinicians a novel tool for noninvasive detection of cancer in dogs. The data presented in this study suggest that real-world use of next-generation sequencing–based liquid biopsy testing can deliver test performance (sensitivity and specificity) and clinical performance (PPV and NPV) at levels consistent with expectations initially established in the test's clinical validation study. Periodic analysis and reporting of test performance, as prospectively documented through clinical experience in large numbers of patients, should be encouraged for all laboratories, especially those offering novel diagnostics.

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Supplementary Materials

Supplementary materials are posted online at the journal website: avmajournals.avma.org