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Interlaboratory Evaluation of *Bretziella fagacearum* Molecular Detection Assays to Guide the eDNA Monitoring of Oak Wilt Disease

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ABSTRACT

Oak wilt disease, caused by the fungus *Bretziella fagacearum*, can kill mature red oaks within months of infection, severely affecting biodiversity, landscapes, and industries. The disease, originally only present in the United States, was officially reported for the first time in Canada in June 2023. The aim of this study was to suggest a standardized assay and sample processing method to optimize oak wilt detection both in infection centers and ahead of the disease front. Two previously published molecular assays, a Nested PCR and a TaqMan qPCR, were compared to detect *B. fagacearum* in a variety of samples in a ring trial across five laboratories. Sample types investigated included eDNA from trapped insect vectors (sorted insects and bulk content from traps), infested and healthy oak wood chips, and *B. fagacearum* conidia dilutions. Results demonstrated that both Nested and TaqMan assays can be used for molecular confirmation of oak wilt, and results are reproducible across different labs. There is a general agreement between both detection assays when testing true-positive and true-negative samples. Both methods demonstrated overall good accuracy. The TaqMan assay was more sensitive and detected lower amounts of DNA target. Both tests were 100% specific to oak wood samples, which was the best sample type to use for detection. In general, samples with high Cts were more prompted to yield false negative Nested results. Detecting oak wilt from bulk insect samples was by far more rapid than sorted sap beetles, but resulted in lower detection signals, especially with the Nested assay. The time-period when the insect traps were set up also had considerable influence on detection results. We hope this study helps to formulate guidelines in oak wilt detection and biosurveillance management.

1 | Introduction

Oak wilt, caused by the fungal pathogen *Bretziella fagacearum*, is a vascular disease that can kill mature red oaks (*Quercus* section *Lobatae*) within months of infection (Henry 1944; Bretz 1953; De Beer et al. 2017). The pathogen enters the sapwood of susceptible trees, disrupts xylem vessels, and eventually blocks the flow of water and nutrients, leading to wilting of the leaves and subsequent death of the host (French and Sienstra 1980). The disease primarily affects red oaks, but most oak species are prone to oak wilt to variable degrees (Gibbs and French 1980). The fungus can spread via root grafts, insect vectors, and anthropogenic movement of infested wood (Gibbs and French 1980; Cease and Juzwik 2001). Underground transmission happens when the fungus travels to neighboring trees (up to 15 m away) through naturally occurring root grafts (Gibbs and French 1980). Long-distance transmission is caused by insect vectors, such as *Caplothorax sayi* (formerly *Carpophilus sayi*) and *Colopterus truncatus* that feed on sporulating fungal mats produced between the inner bark and outer sapwood of infected trees. The insect vectors can transport spores over long distances, sometimes as far as 600 m (Shelstad et al. 1991; Juzwik et al. 2008; Jagemann et al. 2018). Movement of mat-bearing firewood can also contribute to the spread of oak wilt (Juzwik et al. 2008).

First reported in 1942 in Wisconsin (Henry 1944; Juzwik et al. 2008), the oak wilt disease is now present in 24 midwestern and eastern states, as well as Texas (EFSA Panel on Plant Health (PLH) et al. 2020). Oak wilt has been at the Canadian border for almost two decades (Jensen-Tracy et al. 2009). Despite strict measures in place and frequent monitoring in regions of interest, oak wilt was finally reported in Ontario, Canada, in June 2023 (Government of Canada 2023). The prior eDNA detection of *B. fagacearum* (Gauthier et al. 2023) and, more worryingly, the official confirmation of the presence of the disease in Canada has raised great concern, making the containment of oak wilt of the utmost importance. Current projections of the costs encountered if the disease was to spread uncontrollably in Canada are estimated to CND$ 350 million for the removal of diseased street trees and replanting, and CND$ 112 million in standing timber value (Pedlar et al. 2020).

Early detection and biosurveillance have proven to be crucial management tools to control and sometimes eradicate plant diseases (Bilodeau et al. 2019; Hamelin and Roe 2020; Luchi, Ioos, and Santini 2020). With oak wilt in particular, survey and treatment when the number of infections is low is more cost-effective and can save a greater number of trees (Horie et al. 2013). Now more than ever, Canadian and US partners will have to work together in order to contain the spread of oak wilt across North America and globally. Although several *B. fagacearum*-specific molecular tests are available (Wu et al. 2011; Yang and Juzwik 2017; Bourgault et al. 2022), currently, there is no consensus on the methodology of choice for the molecular detection of oak wilt.

For instance, many diagnostic laboratories in the US use the Nested PCR method developed by Wu et al. (2011), which has been optimized in various settings. Bourgault et al. (2022) developed a TaqMan qPCR assay which is currently used in Canada for biosurveillance and the detection of *B. fagacearum* (Gauthier et al. 2023). Both approaches provide advantages and shortcomings; for example, the starting material needed to perform those assays varies wildly. Molecular detection confirming the diagnosis of suspected oak wilt-infested trees is performed on eDNA extracted from wilted leaves, fungal mats, or twigs showing xylem discoloration. On the other hand, molecular detection performed to prevent establishment or to monitor spread following establishment and eradication measures is usually achieved by setting up Lindgren traps baited with pheromones to attract insect vectors known to carry *B. fagacearum* spores. Most previous literature of trapping Nitidulidae associated with oak wilt have utilized wind-oriented traps (Dowd, Bartelt, and Wicklow 1992), however DiGirolomo et al. (2020) showed that Lindgren traps performed as good or better for Nitidulidae versus wind-oriented traps while having the advantage of already being in the toolbox of most forest health agencies for their usefulness in capturing other forest pests, such as bark beetles and wood borers. Lindgren funnel traps are also beginning to be utilized for monitoring of fungal pathogens by extracting DNA from the trapping fluid (Bérubé et al. 2022; Gauthier et al. 2023). From there, some will sort the content of the traps to identify and select specific insect vectors, and then perform the assay on individual or small groups of beetles (McLaughlin et al. 2022). This process, though lengthy, has been deemed more sensitive. Another time-effective monitoring approach is to simply set up more traps for a longer period, process the content of the traps as a whole, and use the assay on a subsample of the bulk trap (Gauthier et al. 2023).

However, both assays and monitoring methods have never been compared and validated on the same samples. While attempts have been made to standardize eDNA monitoring in many fields (Gagné et al. 2021; Langlois et al. 2021), those efforts have not been described and adopted for the oak wilt pathogen, *B. fagacearum*. The enormous potential of eDNA methodology in biosurveillance and invasive species detection should be explored and standardized in this context. Therefore, a multi-laboratory ring trial on sample preparation and available detection tests would increase end-user confidence, which in turn would guide the establishment of robust eDNA monitoring programs for the oak wilt pathogen.

The overall goal of this study was to compare the most common sampling methods and molecular assays available to detect *B. fagacearum*. To address this, we conducted a ring trial among institutions already contributing to oak wilt diagnostics, biosurveillance, and/or research. A total of five laboratories tested DNA samples of different types (sorted insects and bulk content from traps, infested and healthy oak wood chips, and *B. fagacearum* conidia dilutions) using the Nested PCR and TaqMan qPCR assays. Results were compiled and analyzed to validate currently available methods to detect oak wilt, and potentially improve the robustness of biosurveillance programs.

2 | Material and Methods

2.1 | Sample Preparation

2.1.1 | Insect Samples

To compare two different sampling methods, six Lindgren funnel traps were set up in an active oak wilt infection center in Crystal Falls (Michigan, USA). Each trap was baited with

fermenting bread dough, as well as pheromones for *C. sayi* and *C. truncatus* as described before (Gauthier et al. 2023). Collection cups were filled with 70% ethanol. The traps were baited at four predetermined time-points between May and July 2022, and on average 3 to 4 days later, the content of all traps was transferred to secure plastic containers and shipped to the US Forest Service, in Durham, New Hampshire, USA. The insects, debris, and collection fluid from the Lindgren traps were strained through a sieve and washed with 70% ethanol to remove dirt and other small particles. Large particles such as conifer needles and leaves were manually discarded. Trapped insects of the Family Nitidulidae (Order: Coleoptera) known to carry *B. fagacearum* spores were identified under a stereomicroscope and the first three specimens were pooled in a 2 mL Eppendorf tube and labeled as "sorted insects". The rest of the insect specimens were identified, numbered, and pooled into 10 mL glass vials labeled as "bulk content" (see Tables S1 and S2 for the number of insects per trap in time, including their taxonomic names). Both sets of samples (sorted and bulk) were air-dried overnight and stored at -20°C until shipment to Canada.

2.1.2 | Oak Wood Samples

Diseased and healthy oak wood DNA samples were obtained from the Plant and Pest Diagnostics Laboratory of the Michigan State University, East Lansing, USA. Healthy oak wood served as negative wood samples for both assays and included wood from red (*Quercus rubra*), black (*Q. velutina*), white (*Q. alba*), and swamp oaks (*Q. palustris*). Twigs from red (*Q. rubra*), black (*Q. velutina*), white (*Q. alba*), and swamp (*Q. palustris*) oak trees harboring oak wilt symptoms were used to obtain *B. fagacearum* cultures and acted as positive wood samples. Wood shavings were sampled from both positive and negative wood samples, and DNA was extracted using the QIAamp Fast DNA Stool Mini Kit following manufacturer's instructions (Qiagen, Valencia, CA, USA).

2.1.3 | Spore Dilution Samples

A total of four *B. fagacearum* conidia dilutions (0.1, 1, 10 and 100 spores/reaction) were included in the ring trial to estimate the sensitivity of both assays. Conidia solutions were prepared as described in Bourgault et al. (2022).

A list of all samples used in this ring trial is described in Table 1, including their classification into sample types, number of samples, and expected results.

2.2 | DNA Extraction of Samples

Sorted insect samples were first lyophilized (freeze-dried) for 2 days at −50°C in a FreeZone 2.5 Liter freeze-dryer (Labconco, Kansas City, Missouri, USA). Samples of three beetles were then flash-frozen in liquid nitrogen and homogenized using a 3 mm tungsten bead on a Tissue Lyser Lab Vibration Mill Mixer (Retsch MM300; Newtown, Pennsylvania, USA) for 1 m 30 s at 30 Hz. DNA was extracted from the resulting powder using the QIAamp DNA mini kit (Qiagen, Valencia, CA, USA) following manufacturer's instructions. For bulk insects, samples were first lyophilized for 5 days at −50°C and then ground in liquid nitrogen with a pestle and mortar. Instruments and material were carefully washed and bleached in between samples. DNA was extracted from a 40–50 mg powder aliquot (roughly 200 µL in volume) using the QIAamp DNA mini kit following manufacturer's instructions. DNA was finally quantified using a Qubit 2.0 fluorometer (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). A total of 20 DNA samples from negative bulk trap samples from another study (Gauthier et al. 2023) were included as negative controls. For conidia dilutions, DNA was extracted as described in Bourgault et al. (2022). Additionally, five distilled water samples were included as negative controls. For positive controls, two DNA samples from Nitidulidae carrying *B. fagacearum* spores (Lamarche et al. 2015), and a gBlock segment used in Bourgault et al. (2022) were included.

TABLE 1 | Molecular confirmation of oak wilt across 115 samples tested using Nested PCR and TaqMan qPCR assays by participating laboratories.

Sample type	Number of samples	Expected result[a]
Sorted insects from traps (2022 traps)	24	+/−
Bulk content of traps (2022 traps)	24	+/−
Diseased oak wood chip DNA	30	+
Healthy oak wood chip DNA	5	−
B. fagacearum conidia dilutions (0.1–100 spores)	4	+
Negative controls (bulk content of traps from 2021)	20	−
Negative controls (water)	5	−
Positive controls *B. fagacearum* DNA	2	+
TaqMan specific gBlock control[b]	1	+
Total	115	

[a]Expected result: +, positive control; −, negative control; +/−, test sample for which a diagnostic result had not been previously confirmed.
[b]The gBlock control (100 copies/µL) is a 300 bp fragment encompassing a part of the *B. fagacearum* ITS region (Bourgault et al. 2022) and only served as a positive control for the TaqMan assay since its sequence did not encompass the sequence amplified by the Nested assay primers.

2.3 | Nested PCR and TaqMan qPCR Assays

This ring trial aimed to test the Nested PCR assay published in 2011 (Wu et al. 2011) and the TaqMan qPCR assay published in Canada (Bourgault et al. 2022). A full description of their running conditions is listed in the respective studies mentioned previously. All laboratories participating in this ring trial used the same reagents regardless of their PCR and qPCR instruments. The PCR instruments included the Applied Biosystems GeneAmp PCR System 2700 and the Biorad T-100 Thermal Cycler. The qPCR instruments were the 7500 Fast Real-time PCR, the BioRad CFX96, the QuantStudio 3, QuantStudio 6, and theViiA7 systems. For the Nested PCR assay, all participants used the same 200 nM primers (Integrated DNA Technologies, Coralville, IA, USA), ddH_2O, and GoTaq MasterMix 2X (Promega, Madison, WI, USA) for both PCR rounds. Participants were free to use the method of their choosing to identify bands on gels, either a traditional agarose gel or capillary electrophoresis such as the QIAxel system (Qiagen). For the TaqMan assay, all laboratories used the same 600 nM primers (Integrated DNA Technologies), 100 nM probe (Integrated DNA Technologies), ddH_2O, and the Quantitect NoROX MasterMix 2X (Qiagen, Valencia, CA, USA). A description of both assays including sequences, duration, and criteria for positivity are shown in Table 2.

2.4 | Ring Trial

The 115 DNA samples composing the panel were aliquoted and randomized by one scientist to create a double-blinded trial between the five participating laboratories. All reagents (primers, probe, double distilled water, and master mix for the Nested PCR and the TaqMan qPCR) were pre-packaged and distributed along with the DNA samples to each laboratory. Total numbers of samples per category slightly varied on a few occasions since some samples could not be run (sample lost in transit, reagents exhausted, analysis impossible, etc). Participants were given instructions to perform both tests and a spreadsheet to enter their results. Every laboratory sent its results back via email, and data was compiled and analyzed by one individual.

2.5 | Statistical Analysis

Analysis was conducted using program R (R Core Team 2023). Heat maps were constructed using the *heatmap.2* function from the *gplot* package (Warnes et al. 2022). Respective performances of both assays were evaluated by constructing confusion matrices for each laboratory, using only samples with a known expected result. Confusion matrices can help evaluate a new technique compared to a reference, delineating the number of true positives, false positives, true negatives, and false negative (Forbes 1995; Cabot and Ross 2023). Various parameters can be measured, including accuracy (a measure of correct predictions), sensitivity (a measure of the proportion of true positives correctly identified), and specificity (a measure of the proportion of true negatives correctly identified) of each assay (Venette, Moon, and Hutchison 2002). Confusion tables were generated and McNemar's *p*-value was calculated using the *confusionMatrix* function from the *caret* package (Kuhn 2008). Pearson chi-square test and pairwise proportion tests were respectively conducted with the *chisq_test* and *pairwise_prop_test* functions of the *rstatix* package. Binomial logistic regressions were performed using *glm* function of the *stats* package. Post hoc pairwise comparisons between laboratories for both assays were done using *emmeans* function of the *emmeans* package. The probability of detecting oak wilt, defined as the proportion of samples known to contain *B. fagacearum* that tested positive with the corresponding assay, was calculated manually. Proportion stacked bar plots were generated with the *ggplot* function from the *ggplot2* package (Wickham 2016).

3 | Results

Overall results for each sample assayed by each laboratory for both methods are displayed in Figure 1 and Table S3.

TABLE 2 | Nested PCR and TaqMan qPCR markers and protocols used for sample assays.

			Nested PCR assay		TaqMan qPCR assay
Target			**ITS**		**ITS**
Primers sequences	PCR#1	ITS-1F	5′-TCCGTAGGTGAACCTGCGG-3′	Cfag ITS F 75-97	5′-TAAAACCATTT GTGAACATACCA-3′
		ITS-4	5′-TCCTCCGCTTATTGATATGC-3′		
	PCR#2	CF01	5′-GGCAGGGACTTCTTTCTT-3′	Cfag ITS R2 215-43	5′-TGAAAGTTTTAACT ATTTTGTTAAATGCA-3′
		CF02	5′-AAGGCTTGAGTGGTGAAA-3′		
Probe sequence			—	Cfag ITS T RC 126-50	6FAM-AACATCCCCTGA AGAAAGAAGTCC-ZEN
Master Mix			GoTaq MasterMix (Promega)		Quantitect No-ROX MasterMix (Qiagen)
Duration time (min)[a]			385		165
Criteria for positivity			A band at 280 bp[b]		Cts for all triplicates < 40[c]

[a]Total duration encompasses reaction setup and running time of each assay until a diagnosis can be made.
[b]A positive signal could include multiple bands (as long as it included one band of the right size), or a faint band of the right size.
[c]For a sample to be positive, all Cts had to be under 40. If even one replicate (out of three) was above 40, the sample was classified as negative.

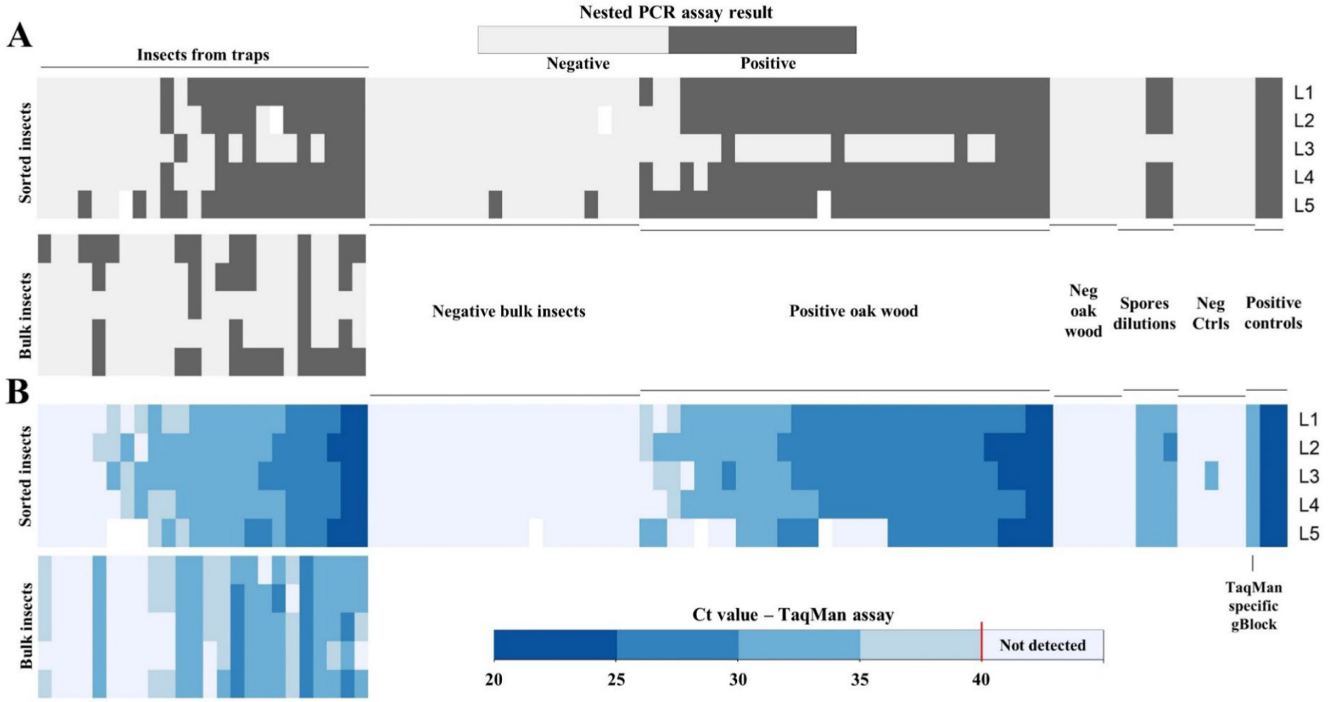

FIGURE 1 | Heatmap of oak wilt detection results using Nested PCR and TaqMan qPCR assays on 115 samples by all laboratories. (A) Top panel shows positive and negative binary results obtained with the Nested assay for all laboratories (L1–L5). (B) Corresponding Ct results for the same samples investigated with the TaqMan assay are aligned in the lower panel. Insect samples from baited traps were divided into sorted and bulk samples and were aligned in paired samples on the left. Samples were grouped into types: Insects from traps, negative bulk insects, positive oak wood, negative oak wood, conidia dilutions, negative controls, and positive controls. Samples were also sorted in ascending order within each category (based on sorted insects for insects from traps), according to Cts obtained for the TaqMan assay. Missing data are represented by white squares.

3.1 | Oak Wilt Detection on Defined Sets of Positive and Negative Samples

Considering only the true-positive samples (culture-validated wood samples, DNA samples from pure *B. fagacearum* cultures, gBlock sample, and conidia dilutions), we observed a general agreement between Nested and qPCR results (Figure 1). Detection results from these true-positive and true-negative samples run with each assay by all laboratories are organized in confusion matrices (Table 3). All laboratories correctly identified the positive controls (*B. fagacearum* DNA and gBlock) with both methods (Figure 1).

Identification and detection of diseased oak wood chip samples was generally acceptable with both methods, except for one laboratory (L3). Their method failed to identify positive samples using the Nested assay (number of undetected samples = 23/30) (Figure 1). However, the remaining 8 false-negative results obtained with the Nested assay represent the same 4 oak wood samples (sample ID: 69, 70, 71, and 73; see Table S3), which also scored very high Cts, and were missed by at least 2 laboratories (Figure 1). A similar pattern was observed with the TaqMan assay, where again 4 positive oak wood samples showed up as false negative (sample ID: 69, 70, 71, and 72; see Table S3) on 7 occasions, with high Cts, hence a very low target DNA concentration (Figure 1).

Compared to Nested PCR, the qPCR TaqMan assay was more sensitive and detected a lower amount of target DNA (1 conidium detected by all 5 laboratories with the qPCR compared to 10 conidia for the Nested) (Figure 1 "Spore dilutions"; Table S3, sample ID 104–107).

When both tests were performed on true-negative samples, including negative DNA from bulk insect traps, negative oak wood samples, and negative controls (water) (Figure 1), we observed a general concordance between Nested and qPCR assays. There was only one instance of cross-contamination for L3 using the TaqMan assay on water, resulting in a false-positive signal. A total of two false-positive detections also occurred with the Nested PCR assay for L5 on negative insect trap samples, however, the bands observed on the gel were extremely faint on both occasions (results not shown).

Confusion matrices are displayed in Table 3. In general, the accuracy for each assay was acceptable, with values all above 0.92 except for L3 and L5 who struggled with the Nested and TaqMan assays, respectively. The sensitivity of the TaqMan assay surpassed the Nested assay for 4 out of 5 laboratories. As expected, the sensitivity was much lower for L3 and L5 for the Nested and TaqMan respectively. Those two laboratories were also the only ones with false-positive detections, resulting in specificities lower than 1 on both occasions. The McNemar's test *p*-values were consistent with all these findings and were lower than 0.001, thus significant, only for L3 (Nested) and L5 (TaqMan), meaning that those detection results were too different from the expected results.

TABLE 3 | Confusion matrices of oak wilt detection based on pooled data for all true-positive and true-negative samples using two molecular methods conducted by participating laboratories.

Lab	Test		Expected result: Negative	Expected result: Positive	Accuracy	Sensitivity	Specificity	McNemar's test *p*-value
L1	Nested PCR	Negative	30	4	0.94	0.89	1	0.134
		Positive	0	32				
	TaqMan qPCR	Negative	30	2	0.97	0.95	1	0.480
		Positive	0	35				
L2	Nested PCR	Negative	29	5	0.92	0.86	1	0.074
		Positive	0	31				
	TaqMan qPCR	Negative	30	1	0.99	0.97	1	1
		Positive	0	36				
L3	Nested PCR	Negative	30	27	0.59	0.25	1	<0.001
		Positive	0	9				
	TaqMan qPCR	Negative	29	3	0.94	0.92	0.97	0.617
		Positive	1	34				
L4	Nested PCR	Negative	30	5	0.92	0.86	1	0.074
		Positive	0	31				
	TaqMan qPCR	Negative	30	3	0.96	0.92	1	0.248
		Positive	0	34				
L5	Nested PCR	Negative	28	2	0.94	0.94	0.93	1
		Positive	2	33				
	TaqMan qPCR	Negative	29	9	0.86	0.74	1	<0.001
		Positive	0	26				

Note: Analysis run only on samples with a known expected result: positive oak wood ($n=30$), positive DNA controls (Nested: $n=2$, TaqMan: $n=3$ with gBlock), positive conidia dilutions ($n=4$), negative oak wood ($n=5$), negative bulk insect traps ($n=20$), negative PCR controls (water: $n=5$).

3.2 | Interlaboratory Variation/Comparison

The complete data set was also analyzed in order to compare variation of results between laboratories. There was no significant difference in the proportion of positive/negative detection among laboratories for the TaqMan assay ($\lambda^2_{\text{Pearson}}(4)=2.62$, $p=0.62$). However, the same analysis for the Nested assay showed significantly different proportions of positive/negative detection among laboratories ($\lambda^2_{\text{Pearson}}(4)=44.41$, $p=5.27\times10^{-9}$). The pairwise comparison showed that the Nested assay results from L3 were different from all other laboratories. It was therefore decided to exclude L3's results for the Nested assay since outlier data would skew subsequence analysis.

3.3 | Comparison of Nested and TaqMan Assays

Overall, the average probability of detecting oak wilt with the TaqMan assay was 91.8 ± 4.5%, while it was 93.8 ± 4.5% with the Nested PCR (Table S4). Of note, L5 was able to get 100% detection rate instead of 74% when using their own in-house TaqMan mastermix (Quantabio PerfeCta ToughMix, Quantabio, MA, USA) on positive oak wood samples (data not shown). Results were also obtained two times faster with the TaqMan assay than with the Nested (165 vs. 385 min, Table 2).

In general, samples with high Cts were more prompted to yield false negative Nested results (Figure 2). Using only samples for which a TaqMan positive signal was obtained, a binomial logistic regression was constructed and showed the probability of Nested PCR detection related to TaqMan Ct value.

3.4 | Oak Wilt eDNA Detection From Field Samples, Either as Sorted Sap Beetles or Bulk Trap Content

In a biosurveillance context, it is common to capture insect vectors in traps located in a region of interest. This comes in useful when trying to detect oak wilt before the appearance of symptomatic trees, to monitor the disease in advance of the front or simply to confirm eradication. One of the goals of this study was to determine if manually sorting the insects from the traps before analysis was worth the effort invested.

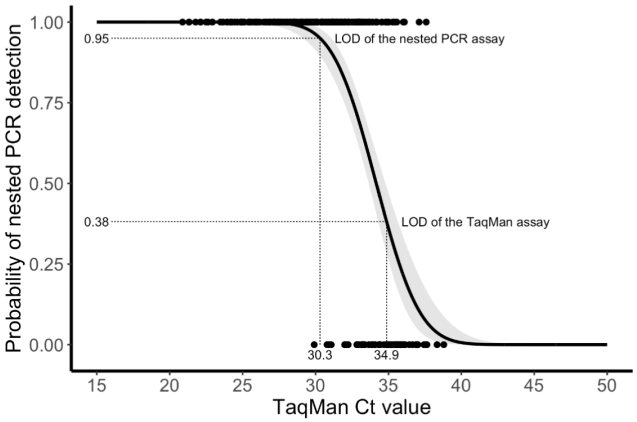

FIGURE 2 | Binomial linear regression showing the probability of Nested PCR detection in function of the TaqMan Ct value. Comparison of results obtained with both assays shows that a theoretical LOD 95% for the nested would correspond to a Ct of 30.3 for the TaqMan assay. There is only a 38% chance of a positive nested signal when reaching the limit of detection for the TaqMan (previously estimated at 34.9; Bourgault et al. 2022). Data include only samples for which a positive TaqMan result was obtained. Results from laboratory L3 were omitted from this data set.

Considering only samples where DNA was extracted from insect traps, there is a general concordance between results with both assays for the sorted insects (Figure 1). However, DNA from bulk insect traps led to a poor performance of the Nested assay. Utilizing Nested PCR assay, a total of 13 sorted insect samples out of 24 were classified as positive by a minimum of three laboratories, whereas only six bulk insect samples came up positive for a minimum of three laboratories (Figure 1A; Table S5). For the TaqMan assay (Figure 1B, Table S5), the total number of positive detections reported by three or more laboratories was 19/24 for sorted insects and 18/24 for bulk insect samples. The five samples that showed up negative for less than three laboratories for the sorted insect samples investigated with the TaqMan were also negative with the Nested assay. Three bulk insect samples (LF-1_bulk, LD-1_bulk, and LB-3_bulk) gave a positive result for the Nested PCR assay, but not their sorted counterpart. A closer examination showed that those three bulk samples actually contained large amounts of sap beetles (respectively 32, 23, and 23 individuals). The TaqMan results were positive for LD-1 and LB-3, sorted and bulk samples alike, but only one laboratory out of five could detect the LF-1 sorted sample as well, whereas all participants were able to detect the LF-1 bulk sample.

The number of beetles per trap varied significantly in bulk samples (ranging from 0 to 32), while there were only two to three beetles in each sorted sample (Table S5). More than 50% of all positive detections reported by a minimum of three laboratories occurred in late June or early July for both assays (Table S6). Our results indicated higher detection rate in late June and early July for both assays and sorting detection methods (Figure 3). However, the TaqMan assay consistently gave a higher proportion of positive detections at every time point, for both sorting methods, except on one occasion where both had a 100% detection rate.

4 | Discussion

In this ring trial study across five different laboratories in the US and Canada, we compared a Nested PCR and a TaqMan qPCR to detect the presence of *B. fagacearum* in multiple environmental sample types.

Our results demonstrated that both Nested PCR and TaqMan assays can be used for molecular confirmation of oak wilt, and results are reproducible across different labs. When performed on various platforms and by different users, both detection methods showed acceptable overall accuracies. The technicians, the chemistry (Master Mix) and the instruments (PCR and qPCR platforms) are variables of the robustness of a test, that is, the capacity of an assay to remain unaffected by minor alterations to test protocols within and between laboratories (Luque-Perez et al. 2013; Waugh and Clark 2021). In this case, for instance, both tests were 100% specific to oak wood samples.

Samples with very low concentrations were harder to detect by every laboratory, for both methods, to various degrees. Approaching the limit of detection of any assay will regularly lead to variability in its detection power (Forootan et al. 2017). For highly concentrated samples of all types, the detection with both assays was usually consistently positive among all participating laboratories. However, the data from this study showed that TaqMan assay is more sensitive than the Nested PCR assay, detecting as low as 1 conidium and predicted to yield confident positives results on samples containing 25 times less target DNA ($2^{\Delta LOD\ Ct\ values}$). Moreover, the TaqMan qPCR assay runs more than two times faster than the Nested PCR and does not require opening and manipulating tubes of amplified DNA for a second round of PCR or gel migration, thus lowering the risks of cross-contamination of samples.

In terms of recommendations for eDNA monitoring of *B. fagacearum* using these assays on different sample types, there are numerous factors that could maximize end-users' confidence in detection results. First, DNA from diseased oak wood is the most reliable sample type to confirm the presence of the oak wilt pathogen. Second, while detecting *B. fagacearum* from bulk insect samples is by far more rapid, sorting the insects from the traps did increase detection signals with both assays, hence overall confidence in the results. Furthermore, adding such steps provides data about the abundance and diversity of the *B. fagacearum* insect vectors carriers present in samples. The very few instances where detection was positive for bulk insect samples but negative for their sorted counterpart could be explained by a non-homogenous distribution of vectors carrying spores or the sheer abundance of insects carrying few spores in those samples. Third, the time-period chosen to harvest beetles lured in traps in the area also influenced the detection of oak wilt in samples, both sorted and bulk, as shown here and by other studies (McLaughlin et al. 2022; Gauthier et al. 2023). Sampling time is therefore crucial for detection to be representative of the situation, as demonstrated here.

If using insects from traps as starting material, caution should be taken with molecular confirmation since the pathogen could

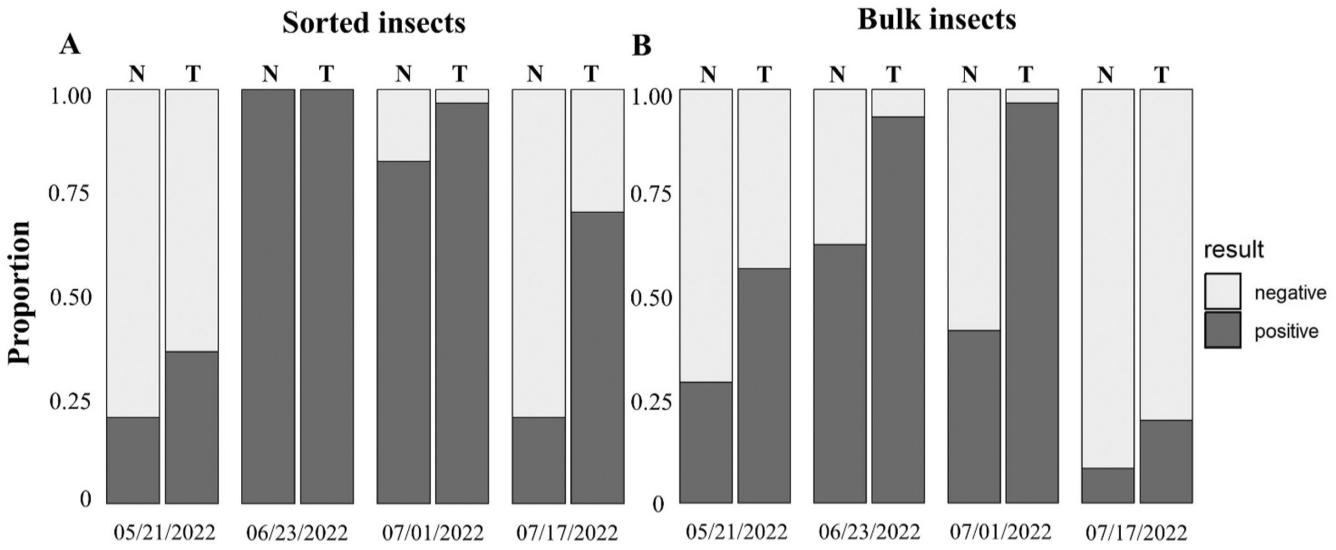

FIGURE 3 | Proportional bar plot graphs showing oak wilt detection in sorted and bulk insect samples over time. (A) Proportion of positive (dark gray) sorted insect samples detected with both assays over time by all laboratories except L3 for Nested assay. (B) Proportion of positive (dark gray) bulk insect samples detected with both assays over time by all laboratories except L3 for Nested assay. N, Nested assay; T, TaqMan assay.

be present but still go undetected when sampling is completed during the off-peak season (ex: early April or late October, depending on the vector investigated). A sampling method incorporating a temporal component (i.e. before and after local insect vector emergence peaks), could have a better likelihood of discriminating eDNA detection of true and false positives, as well as negatives. While focusing the sampling at the sap beetle emergence peaks appears reasonable, eDNA detection could also benefit from an extended sampling period since McLaughlin et al. (2022) reported positive detections later in the season (July–November). Moreover, increasing the number of traps per location improves detection odds, especially when working outside of Nitidulidae emergence peak periods, or if those are unknown. Using three traps per site, McLaughlin et al. (2022) identified *B. fagacearum* with the Nested PCR assay in 10 out of the 13 investigated sites located outside known infection centers over a three-year period. All these new positive sites were located beyond the expected dispersal range of the vectors, suggesting the presence of cryptic diseased trees in the vicinity of the sampling sites rather than the movement of insect carriers from known infection centers. In our study, very few traps were positive at time-points located outside the detection window to which these assays and trapping methods are most sensitive. Therefore, setting up any less number of traps would have resulted in a negative signal. Furthermore, the traps were purposefully set up in an active oak wilt infection center. Hence, in a context of early detection, there would likely be even lower counts of infected beetles if trapping occurred outside of known oak wilt areas.

To complement robust detection tests and appropriate sampling structures, regular proficiency testing would benefit both research and diagnostic laboratories. It is essential to validate and, if necessary, improve the capacity of laboratories and their personnel to detect oak wilt successfully and reliably. In this study, some of the laboratories may have been less familiar with one of the two techniques assayed, as shown by the necessary exclusion of Nested results from L3. In hindsight, this ring trial could have benefited from a short practice round with 5–10 samples first, supported by a more descriptive protocol to set up parameters and interpret the results. With one laboratory failing the Nested assay and another not quite performing optimally with the TaqMan, a prior proficiency panel could have verified technicians' expertise and confirmed instrument compatibility. This has been done before, for example, during an interlaboratory ring trial evaluating the reliable detection of SARS-CoV-2 (Mills et al. 2022). Additional testing with different master mixes could also investigate the influence of this factor on the robustness of the assays. Since the sensitivity and specificity of a real-time PCR assay may vary depending on master mix and platform used, the best combination of chemistry and instrument must be determined (Kang et al. 2021). Human factors leading to false-positive detections, including inexperience, poor bench working methods, or chronic contamination of laboratory equipment with *B. fagacearum* DNA could also be addressed with such a panel. Indeed, laboratories throughout the world applying for official certifications for disease diagnostics or various international quality standards, for instance, must first perform proficiency testing panels (Johnson and Cabuang 2021).

Considering current oak wilt outbreaks in parts of the US and its recent first report in Canada (Government of Canada 2023), the need for a rapid, reliable, and standardized method to identify *B. fagacearum* is even more pressing. Ring trials such as this one usually act as a first step toward an effective global surveillance program, from which everyone can benefit. As seen in this study, additional efforts will have to be made in concert with all laboratories to develop guidelines for eDNA biomonitoring of oak wilt with both assay protocols, especially for the lower concentration samples.

Criteria have been proposed before to report eDNA qPCR results in numerous contexts, where the DNA target is typically

in low-abundance, or when a non-invasive detection approach must be used to reduce disturbance to species and their ecosystems (Nicholson et al. 2020; Beng and Corlett 2020; Gagné et al. 2021; Langlois et al. 2021). For instance, clear guidelines have been established for study designs, how to choose sampling areas, station positions, and number of field sample replicates and laboratory technical replicates. Inclusion and types of positive and negative controls, methods of inhibitor detection, and information on sensitivity and specificity have been discussed. More importantly, decision criteria to determine positive and negative samples as well as threshold settings are among key components when reporting results (Langlois et al. 2021). In the case of oak wilt detection, the cost of incorrectly inferring the absence of the pathogen would be very high and could cause irreversible damage. Therefore, setting a lower detection threshold might be beneficial. Others have also used these guidelines in their work, such as Chevrinais and Parent (2023), who actually created a decision tree compiling criteria for reporting eDNA detection results of the Atlantic wolffish (*Anarhichas lupus*) from their qPCR assay, sorting results as positive, negative and inconclusive.

To this date, there are many options for *B. fagacearum* detection, going from visual inspection of symptoms and conventional microscopy to molecular detection by different techniques. This study managed to formulate recommendations for the eDNA monitoring of oak wilt, showcasing advantages and shortcoming of Nested and qPCR assays currently in use, as well as the benefits of sorting insects from traps and processing them as bulk. However, efforts must be maintained to constantly evaluate new detection methods and sampling strategies that are developed around the world, in order to better control the spread of the disease. As such, this work laid the foundations for future ring trials to compare breakthrough technologies, share knowledge between scientists, and contribute to the fight against oak wilt.

Author Contributions

P.T., D.H., K.C.M., I.M., M.F.D., and T.L.B. contributed to the conception and design of this study; A.D., M.O., K.L.S.-C., L.M., and G.J.B. acquired the data during the ring trial; M.-K.G. and P.T. compiled and analyzed the data; M.-K.G. and P.T. wrote the manuscript. All authors have revised and approved the final version of this manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The authors have archived the raw data underlying the main results of the study in Appendix S1.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.