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# Development and evaluation of a recombinase polymerase amplification assay for rapid detection of strawberry red stele pathogen

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## Abstract

*Phytophthora fragariae* causes drastic damage in strawberry crops. *P. fragariae* infects strawberry roots and causes red stele root rot. Although *P. fragariae* is a quarantine organism, its spread in Finland continues as more and more fields contract the disease. The spread can be halted through developing rapid and reliable detection assays. We have developed a rapid recombinase polymerase amplification (RPA) assay for *P. fragariae* targeting the *Phytophthora* mitochondrial DNA intergenic *atp9-nad9* marker. The assay is DNA-extraction free and capable of detecting as low as 10 fg of *P. fragariae* genomic DNA. We found the assay reliable for diagnosing field plants when samples are adequately collected. We also applied the RPA assay to the detection of the pathogen in the soil through coupling the assay with baiting with the host plant. The results suggest that if only a small number of samples are analysed, the baiting results will not be reliable.

**Keywords:** *Phytophthora fragariae*, Red stele, Recombinase polymerase amplification, Crude maceration, Baiting, Indicator plants

## Background

*Phytophthora fragariae* is a quarantine organism (A2 list of EPPO) and causes red stele root rot of strawberry. This disease is common in regions with cool and moist climates, with damage more extensive in heavy saturated clay soils and during early or late summer. The pathogen first destroys fine roots and later progresses upwards in the stele of larger roots. Plants wilting usually first appears in low or poorly drained areas of fields and the affected area progressively widens over time. When plants with early wilting symptoms are dug out, their major roots have less lateral roots and present a ‘rat-tail appearance’. Dissecting major roots upwards displays abnormal reddish colour in internal cores known as ‘red

stele’ and considered as a typical diagnostic feature of the disease. The disease has drastic nature and causes wilting, stunted growth, less or no strawberries and few stolons. Moreover, the pathogen can persist at least ten years in infested soils through its survival form of oospores (Maas, 1998; Ellis, 2008; Newton et al. 2010).

The disease has badly damaged strawberry crops in North America, Switzerland, Germany, France and Sweden (Maas 1998). *P. fragariae* was not present in Finland according to the EPPO/ CABI, 1997. The absence of the pathogen was also confirmed by the countrywide survey for red-stele disease conducted on strawberry roots collected in 1995 (Pohto 1999). However, in 2012 EVIRA (Finnish Food Safety Authority) surprisingly detected *P. fragariae* for the first time among 55 of the strawberry plantations collected from outdoor horticultural production in different regions of Finland (EVIRA 2013). In North Savo region of Finland, we have observed crop

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damages (loss of plants) of up to 90%, while we have diagnosed 17 fields with red stele among the 42 problematic fields investigated in summer 2017 and 2018. Strawberry crop has a unique importance in Finland. Out of the total 19,131 ha open area cultivated during 2018 in Finland, 4160 ha area produced strawberry with a total yield over 15 million kg (Niemi and Väre 2019).

Common techniques employed for *Phytophthora* diagnosis include symptom observation, microscopy, baiting, immunological assays, and polymerase chain reaction (PCR) (Martin et al. 2012). Duncan (1976) employed susceptible alpine strawberry as bait to quantify the pathogen in infested soils. The bait plants *Fragaria vesca* clone VS1, and *Fragaria vesca* var. *alpina* (Baron Solemacher) were planted with different dilutions of soil and kept at 15 °C for 5 weeks. After 5 weeks, bait plants were examined for red steles and oospore presence. In another article, Duncan (1980) presented a root-tip bait method for the detection of red stele disease in stock plants. In this method, root tips were collected from stock plants and mixed with compost. Then the tips-compost mixtures were added to pots and planted with bait plants *Fragaria vesca* var. *alpina* (Baron Solemacher). Plant pots were watered every 6 h through mist irrigation, and the temperature was maintained at 15 °C. After 5 weeks, bait plants were examined for red steles and oospore presence. The root-tip baiting was reported sufficiently sensitive with capability to detect even one infected plant among 99 healthy ones.

For immunological diagnosis, antisera raised against mycelia of *Phytophthora fragariae* showed cross-reactivity with other *Phytophthora* and *Pythium* species (Amouzou-Alladaye et al. 1988; Werres 1988; Mohan 1989). In contrast, PCR development has been successful over time. The foremost PCR assays developed for the detection of *P. fragariae* utilized the multicopy internal transcribed spacer (ITS) region. Stammler and Seemüller (1993) developed a PCR assay from the ITS region of the rDNA for the detection of *Phytophthora rubi* and found the assay equally specific for *P. fragariae*. Later Bonants et al. (1997) found the assay less sensitive and cross-reacting with *Phytophthora citrophthora*, *Phytophthora nicotianae* A1 and *Phytophthora capsici*. Using the ITS region, Bonants et al. (1997) further developed a nested PCR assay, and improved specificity and sensitivity of *P. fragariae* detection. The nested PCR was able to detect the pathogen in symptomless plants with a sensitivity reported down to twenty zoospores. The nested PCR was also coupled with ELISA (enzyme-linked immunosorbent assay), and for the purpose, a capture probe was designed according to an ITS 1 location with maximum basepair difference from other *Phytophthora* species. The PCR-ELISA design resulted in superior specificity. In a separate study, Bonants et al.

(2004) coupled the nested PCR assay with bait test and shortened the baiting time up to 14 days. The study also compared the sensitivity of different PCR formats for the detection of *P. fragariae* DNA. The PCR formats of gel electrophoresis, PCR-ELISA, TaqMan, and Molecular Beacon were reported equally sensitive, all detecting as low as 100 ag *P. fragariae* DNA through nested PCR. In contrast, the DIAPOPS (detection of immobilized amplified product in a one-phase system) format was reported less sensitive with detection limit as low as 10 fg *P. fragariae* DNA through nested PCR.

Besides development of *Phytophthora* species-level PCR assays, the ITS region has also been utilized to develop *Phytophthora* genus-specific PCR assays and those assays were coupled with restriction enzymes or sequencing to identify different *Phytophthora* species including *P. fragariae* (Cooke and Duncan 1997; Ristaino et al. 1998; Camele et al. 2005; Drenth et al. 2006). Single copy genes containing introns have also been employed for *P. fragariae*-specific PCR assays. Ioos et al. (2006) developed single-round PCR assays from *RAS*-like and *TRPI* genes with sensitivity down to 100 fg. Recently, a multicopy mitochondrial marker, the spacer between the *atp9-nad9* genes, with an appropriate level of variation among *Phytophthora* species was used to develop species-specific diagnostic assays. The *atp9-nad9* is a unique marker of the genus *Phytophthora* and it is a highly conserved gene order present in almost all the *Phytophthora* species but absent in the related genus *Pythium*, Eumycotan fungi and plants (Bilodeau et al. 2014; Miles et al. 2015; Miles et al. 2017). With this locus several *Phytophthora* species-specific assays have been developed, including assays for *P. fragariae*: TaqMan qPCR assays (Bilodeau et al. 2014; Miles et al. 2017; Rojas et al. 2017; Hao et al. 2018; Munawar et al. 2020), SYBR Green PCR assays (Munawar et al. 2020), and recombinase polymerase amplification (RPA) assays (Miles et al. 2015; Rojas et al. 2017; Munawar et al. 2019).

Certified commercial plant stock was reported to have been infected with red stele (Duncan 1980). In 2017, we have also found red steles in one of the certified nursery plant boxes imported from another EU member country to the North Karelia region of Finland. *P. fragariae* infection also has a cryptic nature and can be distributed via asymptomatic exported plants. Besides the cryptic nature of the pathogen, disease symptoms may also disappear during warm summer weather (McGrew 1889). Moreover, in the case of extensive field damage, the leftover plants do not always present red steles. For these reasons, a rapid, sensitive, on-site and reliable assay such as RPA is of the utmost importance. In this study we are presenting development of an RPA assay for detection of *P. fragariae* through *atp9-nad9* marker, following our previous assay design (Munawar et al. 2019).

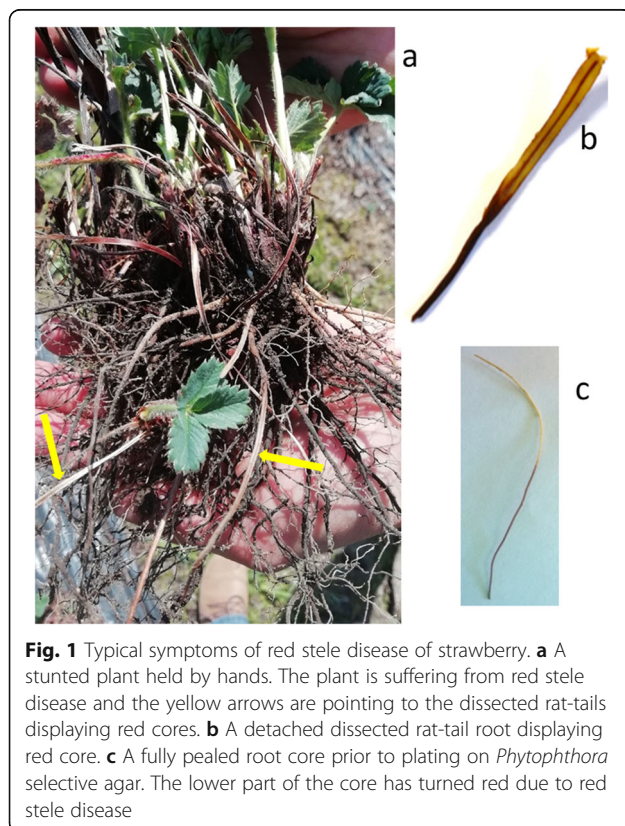
## Results

### Isolation of *P. fragariae* and sequencing of *atp9-nad9* marker

For isolation of *P. fragariae* from infected field plants, we collected red cores, disinfected them with the traditional isolation reagent ‘Teepol L Detergent’ or routine 70% ethanol, and plated them on *Phytophthora* selective agar. Images of an infected plant, rat-tails, and red cores are presented in Fig. 1. We performed isolation trials for 17 red stele-infected fields but succeeded with only three fields. We also compared the traditional Teepol L Detergent disinfection method with the routine 70% ethanol disinfection during isolation trials from three of the infected fields. We found no difference between the two methods in restriction of contaminant growth on the *Phytophthora* selective agar. Moreover, we sequenced the three of our *P. fragariae* isolates (MRV1, VTJ1 and KRT1), the SCRP245 *fvf7* *P. fragariae* isolate, and five *P. fragariae* infected plants, all originating from different fields, and observed unvaried *atp9-nad9* sequence identical to the GenBank accession numbers JF771842, JF771843, or JF771844.

### Optimum RPA assay

Miles et al. (2015) provided partial *atp9-nad9* sequences of more than one hundred *Phytophthora* species to support further development of *Phytophthora* assays. We

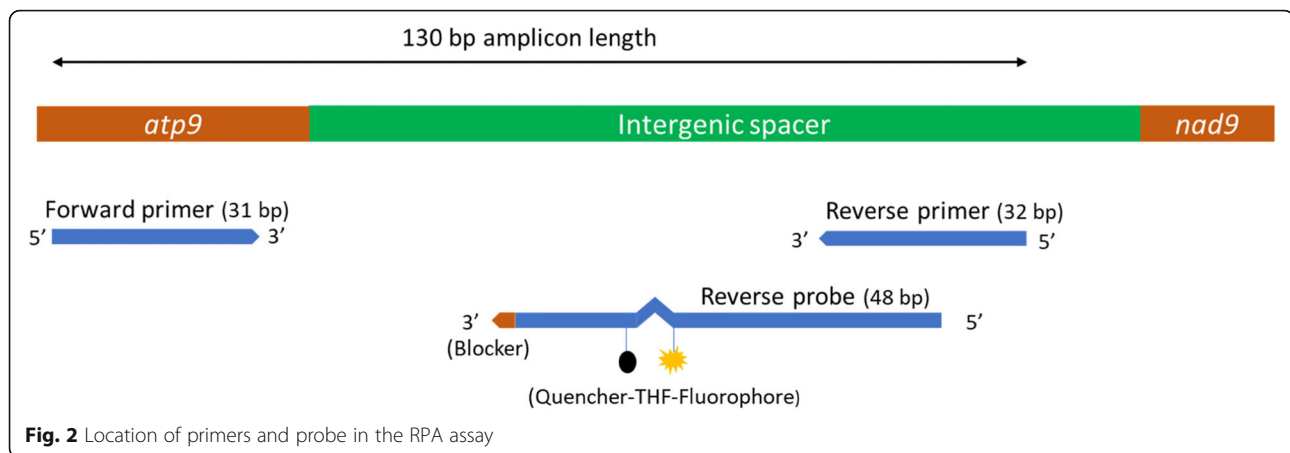


aligned those sequences with Geneious 8.1.9 (Geneious, New Zealand) and recognized the partial *atp9* gene sequence, intergenic spacer, and partial *nad9* gene sequences. We have presented alignment of the *atp9-nad9* sequences, keeping one isolate per *Phytophthora* species (Additional file 1: Figure S1). Subsequently, we designed an RPA assay by picking forward RPA primers from the genus-conserved region of the *atp9* gene, and reverse RPA primers and an overlapping reverse probe (TwistAmp exo probe) from the intergenic spacer between the *atp9* and *nad9* genes. The positions of each primer and probe for the optimized RPA assay are presented in Fig. 2.

The RPA assay was optimum with the forward primer ‘Phy\_Gen\_F3’ at a concentration of 600 nM (3  $\mu$ L of 10  $\mu$ M), reverse primer ‘Phy\_Frag\_R29’ at a concentration of 260 nM (1.3  $\mu$ L of 10  $\mu$ M), and the probe ‘Phy\_Frag\_RevP3’ at a concentration of 100 nM (0.5  $\mu$ L of 10  $\mu$ M). Rest of the components were kept as per manufacturer’s recommendations. Template or macerated sample volume was kept at 1  $\mu$ L. Primer and probe sequences are presented in Table 1.

### Sensitivity and specificity of RPA assay

We evaluated sensitivity of the RPA assay through amplifying 1/10 dilution of *P. fragariae* (MRV1 isolate) genomic DNA ranging from 1 ng/ $\mu$ L to 1 fg/ $\mu$ L. We prepared dilutions of genomic DNA extracted from dried hyphae of *P. fragariae* and quantified with a Qubit 2.0 Fluorometer, utilizing Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific). We found the RPA assay could consistently detect down to 10 fg of *P. fragariae* genomic DNA (five repeats). Amplification curves are presented in Fig. 3. We repeated the amplification of DNA dilutions after spiking individual RPA reaction with 1  $\mu$ L of healthy rootlet macerate. Spiking with healthy rootlets macerate slightly raised baseline fluorescence signal in all tubes and only lessened steepness of 10 fg curve (data not shown). We also compared the sensitivity of the RPA assay in parallel with the *P. fragariae*-specific SYBR Green and TaqMan PCR assays described by Munawar et al. (2020) and Bilodeau et al. (2014). The result showed that the *P. fragariae*-specific TaqMan and SYBR PCR assays are more sensitive with a detection limit down to 1 fg of *P. fragariae* genomic DNA (four replicates for both PCR assays). The RPA assay amplified all of the four *P. fragariae* isolates and presented no amplification with 50–100 pg DNA of *Phytophthora cactorum*, *Phytophthora taxon raspberry*, *Phytophthora megasperma*, *Phytophthora rosacearum*, *Phytophthora ramorum*, *Phytophthora plurivora*, *Phytophthora pini*, *Phytophthora cambivora*, *Phytophthora cinnamomi*, *Pythium sylvaticum*, *Botrytis cinerea*, *Colletotrichum acutatum*, *Mucor hiemalis*, *Fusarium avenaceum*, and *Fusarium proliferatum*. Details of the isolates utilized



in sensitivity and specificity analyses are provided in Table 2.

#### Maceration buffer

Miles et al. (2015) tested different commercial and homemade buffers for their suitability for maceration of plant tissues aimed at RPA, and found GEB 2 (Agdia Inc. Unites States) and standard ELISA grinding buffer suitable for the purpose. We further investigated if all components of the homemade standard ELISA grinding buffer are crucial. We prepared a standard ELISA grinding buffer as 1× phosphate-buffered saline buffer supplemented with 2 g of bovine serum albumin (BSA), 20 g of PVP-40, and 0.5 mL of Tween 20 per litre buffer. We also prepared three modified ELISA grinding buffers, each missing one of the crucial components: BSA-free, PVP-free and Tween-free ELISA grinding buffers. All buffers were prepared without pH adjustment. We collected fine rootlets from naturally infected plants, and pooled, chopped, divided them equally into four 2 mL round-bottom Eppendorf tubes and weighted to prepare 1:10 (w/v) macerates. We macerated three samples with either BSA-free, PVP-free, or Tween-free ELISA grinding buffer and the fourth sample with a standard ELISA grinding buffer. After maceration, we tested supernatants from the four tubes with the RPA assay. We surprisingly found that the three rootlet samples macerated in BSA-free, PVP-free and Tween-free ELISA grinding buffers, and the fourth rootlet sample macerated in standard ELISA grinding buffer (full components) all

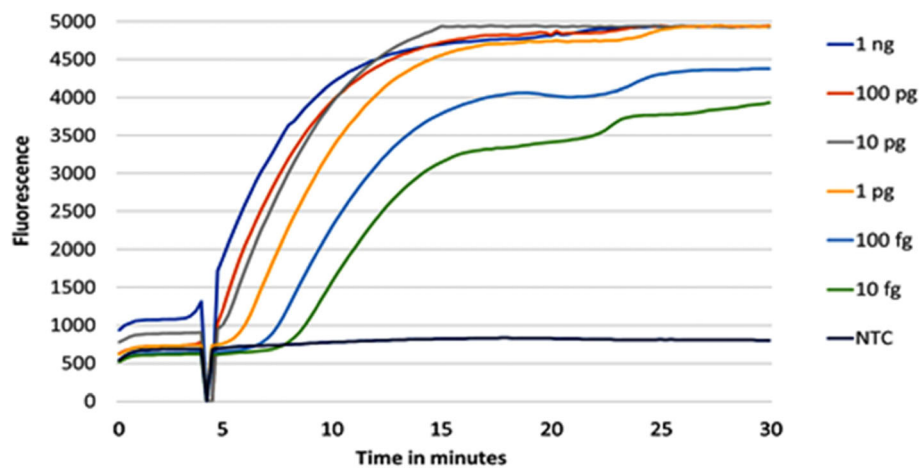
gave equal amplification curves. We repeated the experiment and included a fifth tube in which rootlets were macerated only in autoclaved distilled water. All the five tubes had equal amplification curves with onset of amplifications between 5.0 and 5.5 min.

#### Field validation

In the field validation by Munawar et al. (2020), 22 fine rootlet samples were collected or received from different problematic fields of North Savo region of Finland, crudely macerated in water for doing some RPA assay and also for extraction of DNA for further validation. The DNA samples were analyzed with *P. fragariae*- and *P. cactorum*-specific PCR assays, and a *Phytophthora* genus-specific PCR assay aimed for Sanger sequencing. The validation revealed that among the 22 rootlet samples, nine contained *P. fragariae*, one was co-infected with both *P. fragariae* and *P. cactorum*, and four contained *P. cactorum*. We also analyzed the rootlet macerates prepared by Munawar et al. (2020) in parallel with the *P. fragariae*-specific RPA assay of this study and compared the results. The *P. fragariae*-specific RPA assay of this study gave identical results with the ten rootlet samples containing *P. fragariae*. We also matched the results with plant symptoms and found nine out of the ten positive plants had typical red steles. Detailed results of the field validation are presented in Table 3. RPA results were recorded as time (minutes) of onset of amplification while PCR results are presented as Ct (cycle threshold).

**Table 1** Sequences of the optimal primers and probe for the RPA assay

Primer/ probe	Name	Sequence (5'-3')
Forward primer	Phy_Gen_F3	TGATGGCTTCTTAATTTTATTGCTTTTA
Reverse Primer	Phy_Frag_R29	TGTTTGAAGAGCTAATTACGTATTAATAT
TwistAmp exo probe	Phy_Frag_RevP3	AATTACGTATTAATATACATATATATC-T(ROX)-A-(dSpacer)-T(BHQ2)-ACGAGATTAATATAAT[3'-C3SPACER]



**Fig. 3** Amplification curves of RPA assay for concentrations ranging from 1 ng to 10 fg purified genomic DNA of *P. fragariae*

### Soil testing

To evaluate combination of baiting and the RPA assay for testing soil contamination with *P. fragariae*, we employed three contaminated fields located in North Savo region of Finland and labelled them as A, B and C field. Similarly, we adopted two methods for baiting in the evaluation. In the first method, indicator plants *Fragaria vesca* var. *alpina* (Baron Solemacher), grown from seeds and approximately two months old, were planted in fields A and B for five weeks. The field A was actively suffering from red stele and at a low site, strawberry plants were progressively dying. We planted five indicator plants at the low site. The field B was cultivated with green peas, but it had a history of red stele infection a few years ago. We planted 10 indicator plants randomly across the field. After five weeks, we removed the indicator plants from fields A and B, observed symptoms and tested them with the RPA assay. We found all the five indicator plants from the field A were positive with the RPA assay while only one of them had typical symptom of red steles. Regarding the 10 indicator plants collected from the field B, none of them had red steles, while only one turned positive with the RPA assay.

In the second method of baiting, we utilized baiting units and soil samples from field C. Strawberry field C was destroyed by red stele disease in 2016 and 2017. In summer 2017, the farmer cultivated hemp on half of the field and green peas on the other half. The field C has an inclination, but no particular low or poorly drained site. In spring 2018, we collected eight soil samples from lower and hemp side, and eight soil samples from lower and pea side. We transferred the 16 soil samples into baiting units and also set up two negative and two positive control baiting units. Positive units were added with soil containing artificially infected strawberry roots (from another study), while in negative units, containers were

solely filled with distilled water. On day 7, 10, 14, 21, 35, and 49 we removed three indicator plants or their roots hanging in the water from each baiting unit, pooled them and tested with the RPA assay. On day 7, two of the eight baiting units containing soil from hemp side of the field were positive, while none of the units representing the pea side were positive with the *P. fragariae* RPA assay. Positive controls were also positive for *P. fragariae* on day 7. On the 10th day, positive units among the hemp-related baiting units remained at two, while one unit was positive among the eight baiting units containing soil from pea side of the field. On day 14 results were the same as day 10. On the 21st day, the RPA results remain unchanged for soil from the hemp cropped field, while another unit was positive (total two) among the eight pea-related baiting units. RPA testing on the 35th day (5 weeks) revealed a total of three positive among the eight units containing soil from the hemp side of the contaminated field, while positive units among the baiting units added with soil from pea side of the field remained two. On day 49 results were the same as day 35. The rootlets from negative control units remained negative till day 49. We also noticed darkening of the roots in all positive baiting units after around a week of RPA positivity. The results of soil testing through baiting units are summarized in Table 4.

### Discussion

Designing a species-specific PCR detection assay requires a marker with high discriminatory power, with a superior sensitivity achieved through the use of a multi-copy marker. The ITS region is a high-copy locus widely used for species identification, but it has limited resolution power to discriminate among *Phytophthora* species. Recently, *Phytophthora* species-specific RPA assays targeting the single copy gene *Ypt1* have been developed

**Table 2** Isolates utilized in sensitivity and specificity analyses and their origins

Species	Isolate number	Host	Source/Origin	Result of specificity analysis
<i>Phytophthora fragariae</i>	MRV1	<i>Fragaria ananassa</i>	Isolated from North Savo region of Finland by the University of Eastern Finland	Amplification onset at ~ 5 min
<i>Phytophthora fragariae</i>	VTJ1	<i>Fragaria ananassa</i>	Isolated from North Savo region of Finland by the University of Eastern Finland	Amplification onset at ~ 5 min
<i>Phytophthora fragariae</i>	KRT1	<i>Fragaria ananassa</i>	Isolated from North Savo region of Finland by the University of Eastern Finland	Amplification onset at ~ 5 min
<i>Phytophthora fragariae</i>	SCR245 fv7	<i>Fragaria ananassa</i>	Most probably isolated from United Kingdom	Amplification onset at ~ 5 min
<i>Phytophthora cactorum</i>	PO245	<i>Fragaria ananassa</i>	Isolated from Poland and donated by Grażyna Szkuta from Państwowa Inspekcja Ochrony Roślin i Nasiennictwa	No amplification
<i>Phytophthora taxon raspberry</i>	GE5	Unknown (species also confirmed through <i>atp9-nad9</i> sequencing)	Unknown origin	No amplification
<i>Phytophthora megasperma</i>	GE9	Unknown (species also confirmed through <i>atp9-nad9</i> sequencing)	Unknown origin	No amplification
<i>Phytophthora rosacearum</i>	SO18	Unknown (species also confirmed through <i>atp9-nad9</i> sequencing)	Unknown origin	No amplification
<i>Phytophthora ramorum</i>	Ph426	<i>Rhododendron catawbiense</i> 'Grandiflorum'	Isolated from Finland and donated by Anna Poimala from the Natural Resources Institute Finland (Luke)	No amplification
<i>Phytophthora plurivora</i>	Ph441	<i>Rhododendron</i> 'Marketta'	Isolated from Finland and donated by Anna Poimala from the Natural Resources Institute Finland (Luke)	No amplification
<i>Phytophthora pini</i>	Ph443	<i>Rhododendron</i> 'Capistrano'	Isolated from Finland and donated by Anna Poimala from the Natural Resources Institute Finland (Luke)	No amplification
<i>Phytophthora cambivora</i>	BBA 21/95-K II	<i>Chamaecyparis lawsoniana</i> 'Columnaris'	Isolated from Germany and donated by Sabine Werres from Julius Kühn-Institut (JKI)	No amplification
<i>Phytophthora cinnamomi</i>	BBA62660	<i>Rhododendron</i> sp.	Isolated from Germany and donated by Sabine Werres from Julius Kühn-Institut (JKI)	No amplification
<i>Pythium sylvaticum</i>	ISO-VTJC	<i>Fragaria ananassa</i>	Isolated from North Savo region of Finland by the University of Eastern Finland	No amplification
<i>Botrytis cinerea</i>	ISO-57C	<i>Fragaria ananassa</i>	Isolated from North Savo region of Finland by the University of Eastern Finland	No amplification
<i>Colletotrichum acutatum</i>	PCF753	<i>Fragaria ananassa</i>	Isolated from Belgium and DNA donated by Jane Debode from the Institute for Agricultural, Fisheries and Food Research (ILVO)	No amplification
<i>Mucor hiemalis</i>	ISO-125-MKR	<i>Fragaria ananassa</i>	Isolated from North Savo region of Finland by the University of Eastern Finland	No amplification
<i>Fusarium avenaceum</i>	ISO2-125-MKR	<i>Fragaria ananassa</i>	Isolated from North Savo region of Finland by the University of Eastern Finland	No amplification
<i>Fusarium proliferatum</i>	Tiste Koe 25	<i>Fragaria ananassa</i>	Isolated from North Savo region of Finland by the University of Eastern Finland	No amplification

(Dai et al. 2019a; Dai et al. 2019b; Yu et al. 2019). In contrast, the mitochondrial *atp9-nad9* marker is a multicopy locus and exhibits enough sequence divergence for designing assay on species-level identification of *Phytophthora*. The *atp9-nad9* marker is unique to genus *Phytophthora* but is absent in related genus *Pythium*, Eumycotan fungi and plants (Bilodeau et al. 2014; Miles et al. 2015; Miles et al. 2017). For these reasons, we also targeted the *atp9-nad9* marker for designing *P. fragariae*-specific RPA assay. Although species-level RPA assays were successfully

developed in a wide range of *Phytophthora* taxa by using the *atp9-nad9* marker (Miles et al. 2015; Rojas et al. 2017), RPA assay of this study has only been validated against a limited number of taxa due to limited resources and therefore, we strongly recommend further analysis of specificity with more taxa.

In our RPA primer screening process, we faced the challenge of background amplification that was resolved through redesigning reverse primers from adjacent locations. RPA primers depend on enzymes and proteins for

**Table 3** Field validation results

Rootlet sample No.	<i>P. fragariae</i> -specific RPA assay of this study	<i>P. fragariae</i> -specific SYBR Green PCR assay	<i>P. fragariae</i> -specific TaqMan PCR assay	Sanger's sequencing results	Symptoms
98	Positive Onset at ~ 5 min	30.7 Ct	34.6 Ct	100% identical to JF771842	Wilting, stunting and red steles
99	Positive Onset at ~ 6 min	30.8 Ct	34.4 Ct	100% identical to JF771842	Wilting, stunting and red steles
101	Positive Onset at ~ 6 min	30.7 Ct	34.2 Ct	100% identical to JF771842	Wilting, stunting and red steles
102	Positive Onset at ~ 6 min	30.0 Ct	34.1 Ct	100% identical to JF771842	Wilting, stunting and red steles
105	Positive Onset at ~ 5 min	34.3 Ct	37.0 Ct	100% identical to JF771842	Wilting, stunting and red steles
106	Negative	No Ct	No Ct	No amplification	Stunting
107	Positive Onset at ~ 7 min	34.7 Ct	38.3 Ct	100% identical to JF771842	Wilting, stunting and red steles
108 (co-infected sample)	Positive Onset at ~ 6 min	33.1 Ct	37.2 Ct	100% identical to JF771842	Wilting, stunting, red steles, and typical crown rot
109	Negative	No Ct	No Ct	No amplification	Stunting
110	Negative	No Ct	No Ct	No amplification	Stunting
111	Positive Onset at ~ 7 min	32.5 Ct	36.5 Ct	100% identical to JF771842	Wilting, stunting and red steles
112	Negative	No Ct	No Ct	No amplification	Apparently healthy
114	Negative	No Ct	No Ct	No amplification	Wilting and typical crown rot
117	Positive Onset at ~ 6 min	29.2 Ct	32.7 Ct	100% identical to JF771842	Wilting and red steles
118	Negative	No Ct	No Ct	No amplification	Wilting and typical crown rot
119	Negative	No Ct	No Ct	No amplification	Wilting
120	Negative	No Ct	No Ct	No amplification	Wilting and typical crown rot
121	Negative	No Ct	No Ct	No amplification	Wilting and typical crown rot
122	Negative	No Ct	No Ct	No amplification	Stunting
123	Positive Onset at ~ 8 min	38.5 Ct	40.6 Ct	100% identical to JF771842	Minor wilting only (no red steles)
125	Negative	No Ct	No Ct	No amplification	Apparently healthy
127	Negative	No Ct	No Ct	No amplification	Apparently healthy

their binding with template and limited knowledge is available about the phenomena which makes some RPA primers overly reactive and some primers non-reactive. Moreover, in our RPA assay, the TwistAmp exo probe was designed with ROX as fluorophore and BHQ2 as

quencher, and we observed a slight rise in baseline fluorescence upon addition of macerate. The fluorescence signal rise wasn't observed upon DNA addition. To combat the problem, we reduced the probe concentration from the recommended volume of 0.6  $\mu$ L per reaction to

**Table 4** Soil results from baiting units

Baiting units	Positive units day 7	Positive units day 10	Positive units day 14	Positive units day 21	Positive units day 35	Positive units day 49
<b>Hemp side soil</b>	2/8	2/8	2/8	2/8	3/8	3/8
<b>Pea side soil</b>	0/8	1/8	1/8	2/8	2/8	2/8
<b>Positive control unit</b>	2/2					2/2
<b>Negative control unit</b>	0/2					0/2

0.5  $\mu$ L or sometimes 0.45  $\mu$ L. Labelling the probe with FAM did not have the same problem with increasing fluorescence in our previous *P. cactorum* assay (Munawar et al. 2019), so we recommend labelling the TwistAmp exo probe with FAM/ BHQ1 and utilizing the optimum concentration of probe for improving the assay's sensitivity. Furthermore, a TwistAmp exo probe labelled with FAM/ BHQ1 is less expensive than the one labelled with ROX/ BHQ2.

RPA is a rapid molecular biology technology for amplifying target nucleic acid, and it has potential to expedite plant diagnostics. RPA amplifies target nucleic acid from simple macerates and thereby eliminates the need for time-consuming and problematic DNA extraction. Additionally, RPA runs for only 20–30 min. Besides time-saving aspect, RPA requires minimal laboratory settings. Being an isothermal nucleic acid amplification technology, RPA does not require sophisticated thermocycler and it can be run with simple portable incubation/detection devices at field sites (Li et al. 2019). Regarding the cost of RPA, it is comparable with the standard diagnostic method of DNA extraction followed by TaqMan PCR. The current price of RPA kit 'TwistAmp exo' for 96 samples is 340 euros, while the RPA probe 'TwistAmp exo probe (ROX/ BHQ2)' at 200 nmol size was purchased for 450 US dollars from LGC Biosearch Technologies (United States) in 2017. The probe was enough for several RPA kits. Regarding the cost of the standard diagnostic method, DNA extraction kit 'DNeasy Plant Mini kit' (Qiagen, Germany) for 50 samples is currently sold at 207 euros, and the TaqMan reaction mix 'Luminaris Probe qPCR Master Mix' (Thermo Fisher Scientific) for around 250 reactions is currently priced at 182 euros. We acquired the TaqMan probe (5'FAM/ZEN/3'IBFQ quencher) at the smallest delivery amount (0.5 nmol) from Integrated DNA Technologies (Belgium) at a price of 65 euros.

Regarding maceration, several reagents have been applied to prepare plant crude macerates that can be directly utilized in RPA reaction. Those reagents include TE buffer (Ahmed et al. 2018), NaOH (Karakkat et al. 2018; Qian et al. 2018), standard ELISA grinding buffer (Miles et al. 2015), and commercial buffers GEB (Kumar et al. 2018), GEB 2 (Miles et al. 2015; Li et al. 2017), GEB 4 (Zhang et al. 2014) and AMP1 (Kalyebi et al. 2015; Ghosh et al. 2018) from Agdia, Inc. In contrast, our investigation on standard ELISA grinding buffer revealed that no component of the buffer was essential for obtaining RPA amplification. We repeated the investigations for our previously developed RPA assay targeting *P. cactorum* (Munawar et al. 2019) and found the RPA assay working equally well when crown macerates were prepared with distilled water. The possibility of preparing macerate in water not only simplifies the process but also reduce cost.

Isolation of *P. fragariae* remains challenging even after several decades of pathogen discovery and there is a need for an efficient isolation method. In our project, over a hundred plates failed in the isolation of *P. fragariae* from red stele plants. Moreover, the traditional reagent utilized for *P. fragariae* isolation, Teepol L Detergent (Montgomerie and Kennedy 1983; George and Milholland 1986; Newton et al. 2010), showed no difference when compared with the routine laboratory disinfectant, 70% ethanol, in suppressing the growth of contaminants on the *Phytophthora* selective agar.

Collection of appropriate strawberry plant samples is crucial in red stele diagnosis. As described by Ellis (2008), we observed the red stele disease first appeared in low or poorly drained sites of fields where plants started wilting and stunting, and the affected area progressively expanded. In most of the contaminated field, when we dug out tens of plants with early symptoms of wilting or stunting and dissected several rat-tail roots, we found some red steles. Occasionally we didn't find red stele in contaminated fields and only laboratory findings confirmed red stele disease. Similarly, we observed that within an infected field, plants in well-drained regions of the field remained healthy and even negative with the RPA assay. So, in the cases where affected sites are not present, plant samples should be preferably collected from low or poorly drained areas of fields. Besides appropriate plant sample collection, we recommend utilization of molecular assays when red steles are not obvious. In our observation, fine rootlets generate the most consistent results for molecular assays such as RPA or PCR. Major roots in an infected plant remain negative unless containing red stele. So, sample collection should focus on collecting a maximum of fine roots. Regarding sampling, another challenge is screening the plants already packed for sale or export. In such situations, a few plants should be analysed from each box, but the process is extremely laborious.

Soil testing is generally desired when a field is not under strawberry cultivation. Our results from planting indicator plants in the fields confirm that solely relying on symptoms of the indicator plants can be misleading. For instance, among the five indicator plants planted in the field A's active disease site, all the five had the disease (RPA positives), but only one of the plants exhibited the typical symptom of red stele. Although coupling indicator or bait plants with molecular assay of RPA gave better results, a negative result cannot confirm the absence of the pathogen. Field B had red stele disease a few years ago, and among the 10 randomly planted indicator plants, only one was positive with the *P. fragariae* RPA assay. The indicator plant which caught the disease might be the only one that by chance got planted in a heavily infested site of the field B. We recommend that



indicator plants should be planted in lower or poorly drained sites of a field and any site known for heavy damage during the previous strawberry cultivation. The indicator plants should be planted in early or late summer and well irrigated to provide the pathogen a favoring environment to infect.

Regarding the soil testing, Duncan (1976) potted field soils and their dilutions to bait *P. fragariae* with indicator plants. We found that field soil samples are often inappropriate for potting indicator plants due to their heavy texture. Moreover, preparing soil dilutions is laborious. For these reasons, we developed a simple baiting system. The baiting system coupled with the RPA assay detected *P. fragariae* in some of the soil samples but didn't turn all the soil samples positive within the traditional baiting period of five weeks. While the number of soil samples and duration of baiting are the factors to be considered, the number of years elapsed from active infection may also influence the degree of pathogen survival and reliability of the baiting system. We recommend the collection of several soil samples, especially targeting sites with a history of heavy damage and the poorly-drained ones. Moreover, keeping the experiment temperature low at around 15 °C is crucial for *P. fragariae* infectivity. Finally, special care should be taken during field inspection and sampling to prevent human-assisted spread of *Phytophthora* across fields. Protective measures include changing shoes cover and washing of sample collection tools prior to moving to the next field.

## Conclusions

In this study, we developed a rapid, specific, and sensitive species-specific detection assay for *P. fragariae* utilizing RPA technology and targeting the *Phytophthora* mitochondrial DNA intergenic marker located between *atp9* and *nad9* genes. We believe that our rapid assay can assist in halting the spread of *P. fragariae* and preventing economic losses of red stele disease of strawberry. We also developed a simpler baiting system compared to the traditional Duncan's baiting for detection of the pathogen in soil. Coupling the baiting with molecular assays such as PCR or RPA provides more reliable results.

## Methods

### Isolation of *P. fragariae*

Rat-tail roots were dissected with a scalpel to observe the presence of red cores. Rat-tails with red cores were further peeled and red cores were surface disinfected, dried, and placed on *Phytophthora* selective agar (Drenth and Sendall 2001). The *Phytophthora* selective agar comprised of corn meal agar (Sigma-Aldrich) supplemented with final concentration of 250 µg/mL for ampicillin (Sigma-Aldrich), 25 µg/mL for benomyl (Sigma-Aldrich), 10 µg/mL for pimarinic (Molekula, Germany), 10 µg/mL

for rifampicin (Duchefa Biochemie, Netherlands) and 50 µg/mL for hymexazol (Alfa Aesar). Surface disinfestation of red stele plants was accomplished by incubating in either 70% ethanol or Teepol L Detergent from Stralab Ltd. UK. When using 70% ethanol, infected roots were given a dip. For Teepol, infected roots were first dipped in 50% Teepol L Detergent for 1 min and then dipped in three vials containing autoclaved distilled water for 5–10 min per vial. Following surface disinfestation, roots were surface dried by placing them on filter paper.

### Collection of *atp-nad9* sequences

*P. fragariae* isolates retrieved from different strawberry fields were grown in peptone glucose broth (Unestam 1965) supplemented with pea broth (Zentmyer and Chen 1969) for around a week at room temperature. The proportion of pea broth in the peptone glucose broth was 5–10% (v/v). After growing *P. fragariae* isolates, hyphae were washed and dried overnight in an oven at 45 °C. DNA was extracted by E.Z.N.A. Fungal DNA Mini Kit (Omega Bio-tek, United States). Similarly, fine rootlets were collected from strawberry plants where *P. fragariae* was not isolated and DNA was then extracted with the DNeasy Plant Mini kit (Qiagen) as per manufacturer's recommendations.

The *P. fragariae atp9-nad9* marker was amplified and Sanger sequenced as previously described (Martin and Coffey 2012; Bilodeau et al. 2014). The amplification PCR was performed using DreamTaq Green PCR Master Mix (2X) (Thermo Fisher Scientific, United States) with an annealing temperature of 57 °C. Sanger sequencing was accomplished at GATC Biotech, Germany.

### RPA optimization

Primer screening for specificity included five forward primers, 21 reverse primers, and one probe. The RPA primers were obtained as standard desalted purified DNA from Integrated DNA Technologies, while the TwistAmp exo probe labelled with ROX fluorophore and BHQ2 quencher, and purified with dual HPLC was obtained from LGC Biosearch Technologies. Ready-to-use lyophilised RPA reactions 'TwistAmp exo' were obtained from TwistDx Inc. (United Kingdom). Primer screening was accomplished as per manufacturer's recommendations. For the optimum primer pair, different ratios of forward and reverse primers were further evaluated. *P. fragariae* hyphal DNA of varying concentrations was utilized as template. RPA reaction incubation and fluorescence monitoring were accomplished through T8-ISO (Axxin, Australia) or Mx3000P QPCR System (Agilent, Germany). Reaction strips were incubated at 39 °C for 20 min with a manual agitation at the fourth minute. Results were reported as negative, positive, and intermediate. The absence of

amplification was considered a negative result. Similarly, any amplification curve shorter or less steep than the curve of 10 fg dilution, *P. fragariae* genomic DNA dilution spiked with healthy rootlets macerate, was considered intermediate. Intermediate results were rare, and they were always repeated with new plant samples. Regarding positive result criteria, any amplification curve equal or more in terms of height and steepness compared to the amplification curve of 10 fg DNA was considered a positive. In the T8-ISO settings, it is also possible to set minimum values for curve amplitude (height) and gradient (steepness) as criteria for positive results. We set a minimum gradient threshold at 7.0 mV/s and a minimum amplitude at 3000 mV. We set ROX as the test channel and its LED PWM level at 30%.

#### SYBR green and TaqMan PCR assays

*P. fragariae*-specific SYBR Green and TaqMan PCR assays described in Munawar et al. (2020) and Bilodeau et al. (2014) were utilized in this study. Those PCR assays were also designed from *atp9-nad9* marker. The *P. fragariae* SYBR Green PCR assay was done with forward primer 'Phy\_Gen\_SeqF4' (ACAACAAGAATTAATGAG AACTGC), reverse primer 'Phy\_Frag\_PCRR4' (TTTTTTG TTTGAAAAGAGCTA) and LightCycler® 480 SYBR Green I Master (Roche, Switzerland). The PCR program included a 10 min pre-denaturation at 95 °C and 40 thermal cycles, each cycle comprising 95 °C for 10 s, 58 °C for 20 s and 67 °C for 20 s. The *P. fragariae* TaqMan PCR assay was done with forward primer 'Phy\_Gen\_SeqF4', reverse primer 'Phy\_Gen\_SeqR8' (GGTAAAAT TTGTAATAAATATTGACT), TaqMan probe 'PfraVf\_nad9sp\_TaqMan2' (/56-FAM/ATC TCG TAA /ZEN/

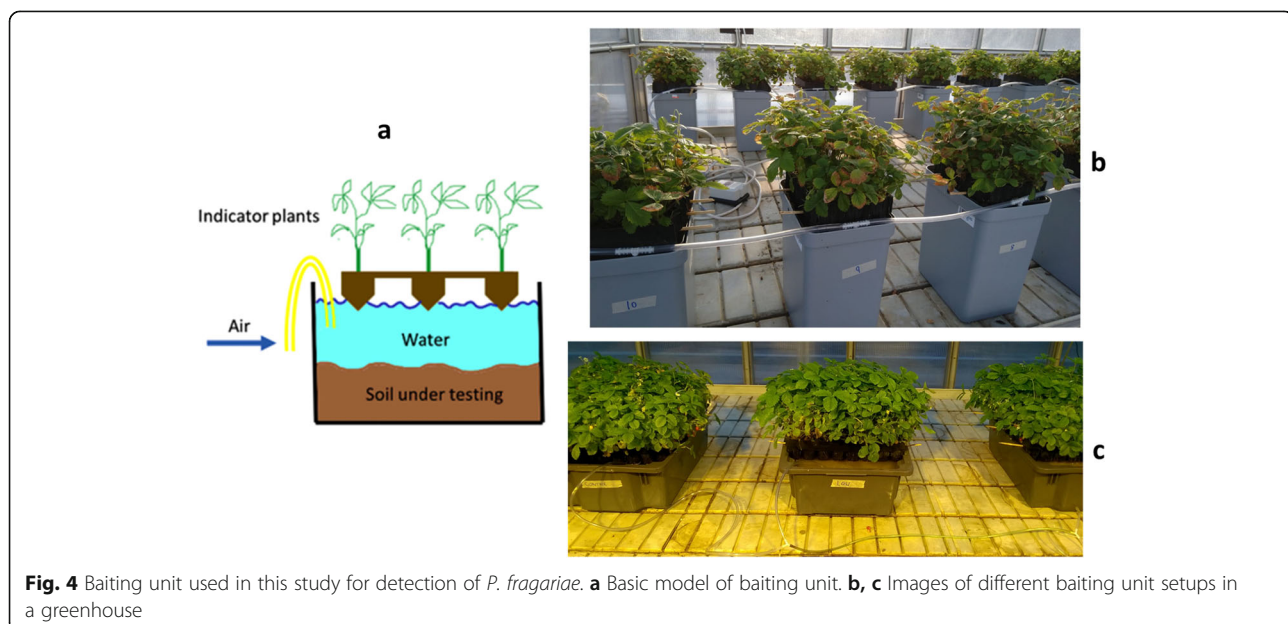
TAG ATA TAT ATG TAT ATT TAA TAC GT/3IABkFQ/) and Luminaris Probe qPCR Master Mix (Thermo Fisher Scientific). The PCR included a 10 min pre-denaturation at 95 °C and 45 thermal cycles, each cycle comprising 95 °C for 15 s followed by 57 °C for 90 s. Each primer concentration was kept at 500 nM while TaqMan probe was 100 nM. Template volume was kept at 1 µL for the 1:10 serial dilution of hyphal DNA and 0.5 µL for plant DNA.

#### Crude maceration

Strawberry plant roots were washed in tap water with a final rinse in autoclaved distilled water. Fine rootlets or root tips weighing between 30 and 100 mg were collected, chopped and transferred into 2 mL round-bottom Eppendorf tubes for maceration with plastic pestles. Maceration was facilitated by initially adding only 100 µL fluid. Following maceration, additional fluid was supplemented to prepare a final 1:10 (w/v) macerate. The macerate was vortexed and left to settle for a few minutes. Then 1 µL of the supernatant was transferred to the RPA reaction.

#### Field soil collection

Soil samples from lower part of the field C were collected from around 3–5 m distances. Soil samples of volume 10–15 L were collected with a shovel from around a 25 cm depth. Soil samples were stored at 4 °C until the baiting experiment was conducted in the autumn of 2018. Before baiting, soil samples were mixed and then five litres of soil from each sample was moved to a corresponding baiting container.



**Fig. 4** Baiting unit used in this study for detection of *P. fragariae*. **a** Basic model of baiting unit. **b, c** Images of different baiting unit setups in a greenhouse

### Baiting unit

A baiting unit comprises a simple container of 10 to 20 L volume. The bottom of the container is first filled with test soil and then an equal volume of distilled water is added. The container is topped with a tray of indicator plants. The tray is a plastic pot block with porous bottom, and it was sown with indicator plant *Fragaria vesca* var. *alpina* (Baron Solemacher) seeds approximately two months prior experimentations. In the baiting unit, the level of the tray was set so that only the porous bottom of the tray was touching the water layer in the unit. The water layer was also aerated through air pump and tubing. Fig. 4 presents model and images of baiting units. The experiments were conducted in autumn or early winter in a greenhouse with the temperature set at 12–18 °C. All the units were filled with distilled water every second day to maintain an adequate water level.

### Supplementary information

**Supplementary information** accompanies this paper at <https://doi.org/10.1186/s42483-020-00069-4>.

**Additional file 1 Figure S1.** Screenshots of Geneious alignment for the *atp9-nad9* sequences of *Phytophthora* species provided by Miles et al. (2015). For the *P. fragariae atp9-nad9* sequence, primers and probe binding regions are annotated.

### Abbreviations

BSA: Bovine serum albumin; CABI: Centre for Agriculture and Biosciences International; DIAPOPS: Detection of immobilized amplified product in a one-phase system; ELISA: Enzyme-linked immunosorbent assay; EPPO: European and Mediterranean Plant Protection Organization; EVIRA: Elintarviketurvallisuusvirasto or Finnish Food Safety Authority; GEB: General Extract Buffer; ILVO: Institute for Agricultural, Fisheries and Food Research; ITS: Internal transcribed spacer; JKI: Julius Kühn-Institut; LUKE: Natural Resources Institute Finland; PVP: Polyvinylpyrrolidone; RPA: Recombinase polymerase amplification

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### Authors' contributions

Most of the lab work was completed by MM. Similarly, the design of the RPA assay was conceived by MM. The design of baiting unit was conceived by HK. The manuscript was written by MM, while all other co-authors have contributed in improving the manuscript. Funding was acquired by HK. All authors read and approved the final manuscript.

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### Availability of data and materials

Not applicable.

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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