On the reliability of recombinase polymerase amplification – lateral flow assay using ITS rRNA gene primers and probe as a new detection method of the golden potato cyst nematode, *Globodera rostochiensis*

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Summary. Recombinase polymerase amplification (RPA) is an isothermal *in vitro* nucleic acid amplification technique, which has been adopted as a novel molecular technology for simple, rapid, reliable, and low-resource diagnostics of nematodes and other damaging organisms. Recently, RPA integrated with lateral flow assay (LFA) using the ITS rRNA gene primers and probe was proposed by Wang *et al.* (2022) as a new detection method of *Globodera rostochiensis*. The reliability of any diagnostic method should be validated on a broad range of populations of different species from different geographical locations as well as on related taxa to avoid erroneous identification with false positive results. A total of 24 isolates of 13 cyst nematode species were used to validate the analytical specificity of the method mentioned above. The results of these tests showed that the ITS rRNA gene primers and probe did not give specific RPA-LFA detection for the golden potato cyst nematode and gave positive reactions not only with *G. rostochiensis*, but also with other *Globodera* parasitising solanaceous plants, *G. artemisiae*, and representatives of the genus *Punctodera* parasitising grasses.

Key words: cross-reactions, *Globodera ellingtonae*, *Globodera pallida*, molecular diagnostics, specific primers, specificity assay.

The worldwide distributed golden potato cyst nematode, *Globodera rostochiensis* (Wollenweber, 1923) Skarbilovich, 1959, is probably the most economically important nematode pest of potato in temperate regions and in some warm areas of the world where short-cycle potato crops are produced in winter months (Inserra *et al.*, 1996). There are estimates indicating that the potato cyst nematodes are responsible for the loss of 9% of this crop worldwide. The golden cyst nematode is recognised as a significant global threat to food security and it is the subject of quarantine regulations in most countries where it occurs (Turner & Subbotin, 2013). The finding of the golden cyst nematode in samples have serious implications for market access and international trade of seed potato. Accurate identification is critical for the implementation of phytosanitary and control measures against this pest.

Recombinase polymerase amplification (RPA) is an isothermal *in vitro* nucleic acid amplification technique, which has been adopted as a novel molecular technology for simple, robust, rapid, reliable and low-resource diagnostics of nematodes and other organisms. RPA represents a versatile alternative to PCR (Tan *et al.*, 2022). RPA assays show high sensitivity and specificity for detecting various pests including nematodes (Subbotin & Burbridge, 2021). Recently, a new detection method of *G. rostochiensis* using RPA integrated with lateral flow assays (LFA) was published by Wang *et al.* (2022). The method allowed the rapid diagnosis and detection of this nematode from crude extracts of cysts and juveniles within 30 min. Moreover, the RPA-LFA method can also directly diagnose and detect *G. rostochiensis* from infested field soil. The authors stated that they designed species-specific

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Table 1. Cyst-forming nematode isolates tested in RPA-LFA assay.

Species	Sample code	Origin	RPA experiment
Globodera rostochiensis	CD2176	Russia, Kaliningrad region, Pravdinskii district	+
G. rostochiensis	CD2598a	Bolivia, Cochabamba, Tapacari, Chuna Chununi	+
G. rostochiensis	CD2197	German, Hannover	+
G. rostochiensis	CD2200a	Germany, Harmerz	+
G. rostochiensis	CD2617a	Bolivia, La Paz, Los Andes, Lacaya	+
G. pallida	CD2740a	The Netherlands	+
G. pallida	CD2553b	Peru, Cusco, Quispicanchis	+
G. pallida	CD2555a	Peru, Cruz Pampa	+
G. pallida	CD2554b	Peru, Cusco, Marangani, Canchis	+
G. tabacum	CD2689a, b	USA, Virginia, Paulette	+
G. tabacum	CD2684a	USA, Virginia, Baines	+
G. mexicana	CD3563a	Mexico, State of Mexico, Juchitepec County	+
G. mexicana	CD3565b	Mexico, State of Tlaxcala, Santiago Cuaula	+
G. ellingtonae	CD3815a	Bolivia, Tarija, El Puente, Chilcayo Sud	+
G. ellingtonae	CD3887a	Bolivia, Tarija, El Puente, Chorcoya Mendez	+
G. artemisiae	CD2178a	Russia, Primorskii Krai	+
Punctodera punctata	CD2715a	USA, Michigan	+
P. stonei	CD3088a	Russia, Jaroslavl region, Myshkin	+
P. stonei	CD2994a	Canada, Ontario, Ottawa Arboretum	+
P. chalcoensis	CD2813	Mexico	+
Cactodera rosae	CD3562	Mexico, Tlaxcala State, Huamantla, Francisco Villa Tecoac	–
C. solani	CD3586a	Mexico, Tlaxcala State, Huamantla, Francisco Villa Tecoac	–
Heterodera avenae	CD2006b	Turkey, Mardin, Midyat, Idil Road	–
H. humuli	CD3144b	Russia, Moscow	–

Note: + – test line; – no test line.

primers and probe, which amplified the internal transcribed spacer of nuclear ribosomal rRNA gene (ITS rRNA) of target nematode (Wang *et al.*, 2022). To determine the specificity in RPA-LFA assay, the authors used the single cyst crude extracts from 14 cyst nematode populations, including nine *G. rostochiensis* samples from different geographical origins, one sample of the pale cyst nematode, *Globodera pallida* Stone, 1973, one sample of *Globodera artemisiae* (Eroshenko & Kazachenko, 1972) Behrens, 1975, and three other cyst nematodes species. The authors stated that the RPA-LFA result showed high level specificity to *G. rostochiensis* and did not detect other potato or other cyst nematode species. Only the strip of *G. rostochiensis* showed both test line and control line, but the other cyst nematode species and negative control showed no test line (Wang *et al.*, 2022).

For validation of any diagnostic methods, the analytical specificity should be assessed by testing a broad range of species of the same taxonomical group, including those from different geographical locations as well as related taxa to avoid erroneous identification with false positive results. The goal of this study was to evaluate primers and probe proposed by Wang *et al.* (2022)

for the *G. rostochiensis* using RPA-LFA detection assay with some representatives of *Globodera* and related genera.

MATERIAL AND METHODS

Nematode samples. DNA was obtained from 24 isolates of 13 cyst-forming nematode species. The nematodes samples included: five isolates of *G. rostochiensis* from Germany, Russia and Bolivia, four isolates of *G. pallida* from Peru and The Netherlands, two isolates of *Globodera tabacum* (Lownsbery & Lownsbery, 1954) Skarbilovich, 1959 from USA, two isolates of *Globodera mexicana* Subbotin, Mundo-Ocampo & Baldwin, 2010 from Mexico, two isolates of *Globodera ellingtonae* Handoo, Carta, Skantar & Chitwood, 2012 from Bolivia, one isolate of *G. artemisiae* from Russia, one isolate of *Punctodera punctata* (Thorne, 1928) Mulvey & Stone, 1976 from USA, two isolates of *Punctodera stonei* Brzeski, 1998 from Russia and Canada, one isolate of *Punctodera chalcoensis* Stone, Sosa, Sosa Moss & Mulvey, 1976 from Mexico, one isolate of *Cactodera rosae* Cid del Prado Vera & Miranda, 2008 from Mexico, one isolate of *Cactodera solani* Escobar-Avila, Subbotin & Tovar-Soto, 2021 from Mexico, one isolate of *Heterodera avenae* Wollenweber, 1924 from Turkey and one isolate of *Heterodera humuli* Filipjev, 1934 (Table 1). All nematodes were identified by molecular method using the ITS rRNA gene sequences (Subbotin *et al.*, 2020).

Sequence analysis. Alignment of the ITS rRNA sequences for *Globodera* and other cyst nematodes were created using ClustalX 1.83 (Chenna *et al.*, 2003) with default parameters using sequences deposited in the GenBank and unpublished sequences (Subbotin *et al.*, 2011, 2020).

RPA-LFA assay. The RPA-LFA assay conducted with the *G. rostochiensis*-specific RPA-LFA primers: GrF4 (5'-CTG TGT ATG GGC TGG CAC ATT GAC CAA CA-3'); GrR4 (5'-[Biotin]TAC GGC ACG TAC AAC ATG GAG TAG CAG CTA C-3'); and probe: GrP (5'-[Fam]CGG AGG AAG CAC GCC CAC AGG GCA CCC TAA CG[THF] CTG TGC TGG CGT CTG T[C3-spacer]-3') (Wang *et al.*, 2022), which were synthetised in Biosearch Technologies (CA, USA). The RPA-LFA assay was done using AmplifyRP® Acceler8® Discovery Kit (Agdia, IN, USA). The reaction mixture for each RPA assay was prepared according to the manufacturer's instructions: the lyophilised reaction pellet was suspended with a mixture containing 6 µl of the rehydration buffer, 2 µl of distilled water, 0.45 µl of each forward and reverse primers (10 µM), 0.15 µl of the probe (10 µM), 0.5 µl of magnesium acetate. One µl of the DNA template was added in a reaction tube. The reaction tubes were incubated at 39°C in a MyBlock Mini Dry Bath (Benchmark Scientific, Edison, NJ, USA) for 20 min. For visual analysis with Milenia® Genline Hybridetect-1 strips (Milenia Biotec GmbH, Giessen, Germany), 120 µl of HybriDetect assay buffer was added to a reaction tube and then a dipstick was placed in this mixture. Visual results were observed within 3-5 min. The amplification product was indicated by the development of a coloured test line (lower), and/or a separate control line (upper) to confirm that the system worked properly.

RESULTS AND DISCUSSION

Lateral flow detection of RPA products obtained using AmplifyRP® Acceler8® Discovery Kit (Agdia, IN, USA) showed positive strong or moderate intensive test lines on the strips with all studied DNA samples of *G. rostochiensis*, *G. pallida*, *G. mexicana*, *G. tabacum*, *G. ellingtonae*, *G. artemisiae*, *Punctodera punctata*, *P. stonei* and *P. chalcoensis*. No test lines were detected with DNA samples of *Cactodera rosae*, *C. solani*, *Heterodera avenae*, *H. humuli* and control without DNA (Fig. 1A & B). Control lines were visualised on all strips. Thus, ITS rRNA gene primers and probe proposed by Wang *et al.* (2022) for the golden potato cyst nematode RPA-LFA detection assay gave positive reactions not only with *G. rostochiensis*, but also with other *Globodera* species from solanaceous plants, *G. artemisiae*, and representatives of the genus *Punctodera* from grasses.

The success of molecular identification depends strictly on the choice of the target gene fragment accounting for possible interspecific sequence variation to avoid strict overlapping with fragments of non-target species. For this aim, the ITS rRNA gene is used widely for diagnostics of cyst-forming nematodes (Subbotin *et al.*, 2011). The ITS region was selected as target gene fragment for diagnostics of *G. rostochiensis* with conventional PCR (Mulholland *et al.*, 1996; Bulman & Marshall, 1997; Zouhar *et al.*, 2000; Trayanov *et al.*, 2020), real-time PCR (Madani *et al.*, 2008; Toyota *et al.*, 2008) and loop-mediated isothermal amplification (Ahuja *et al.*, 2021) with species-specific primers. However, use of this fragment for development of these diagnostic methods is problematic, because of a high sequence identity between species. For example,

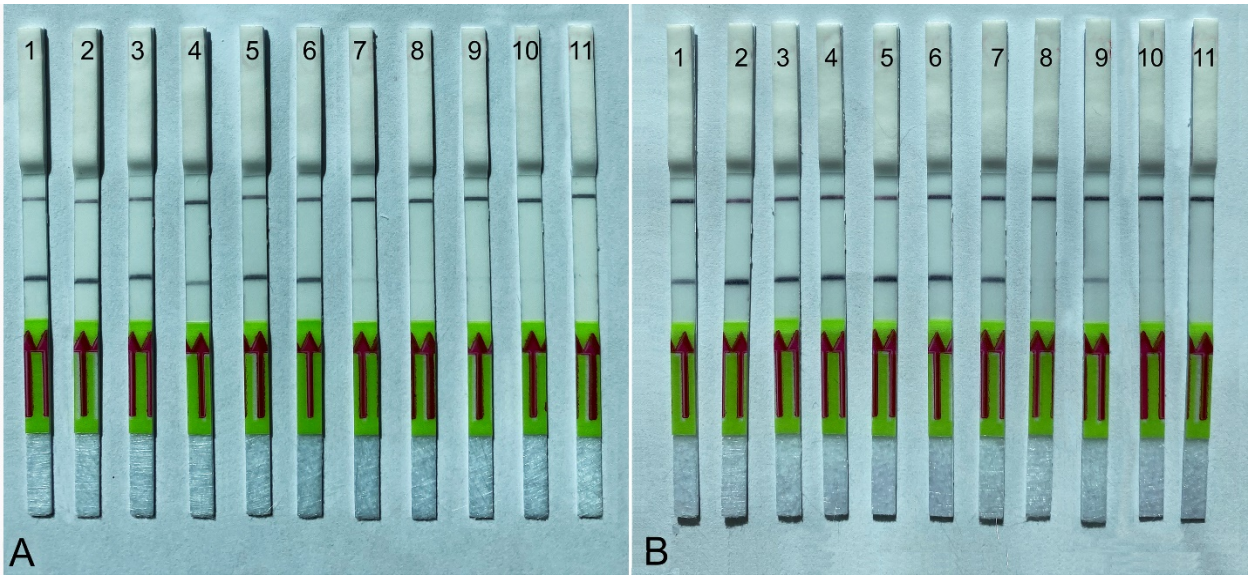

Fig. 1. RPA-LFA specificity assay with examples of lateral flow strips. DNA of cyst-forming nematodes: A. Strips: 1 – *Globodera pallida* (CD2555a); 2 – *G. pallida* (CD2554b); 3 – *G. tabacum* (CD2684a); 4 – *Punctodera chalcoensis* (CD2813); 5 – *P. stonei* (CD2994a); 6 – *G. rostochiensis* (CD2617a); 7 – *Cactodera rosae* (CD3562); 8 – *C. solani* (CD3586a); 9 – *Heterodera humuli* (CD3144b); 10, 11 – NC (control without DNA). B. Strips: 1 – *G. artemisia* (CD2178a); 2 – *G. tabacum* (CD2689b); 3 – *P. punctata* (CD2715a); 4 – *P. stonei* (CD3088a); 5 – *G. mexicana* (CD3563a); 6 – *G. rostochiensis* (CD2200a); 7 – *G. tabacum* (CD2689a); 8 – *H. avenae* (CD2006b); 9 – *G. pallida* (CD2553b); 10, 11 – NC (control without DNA).

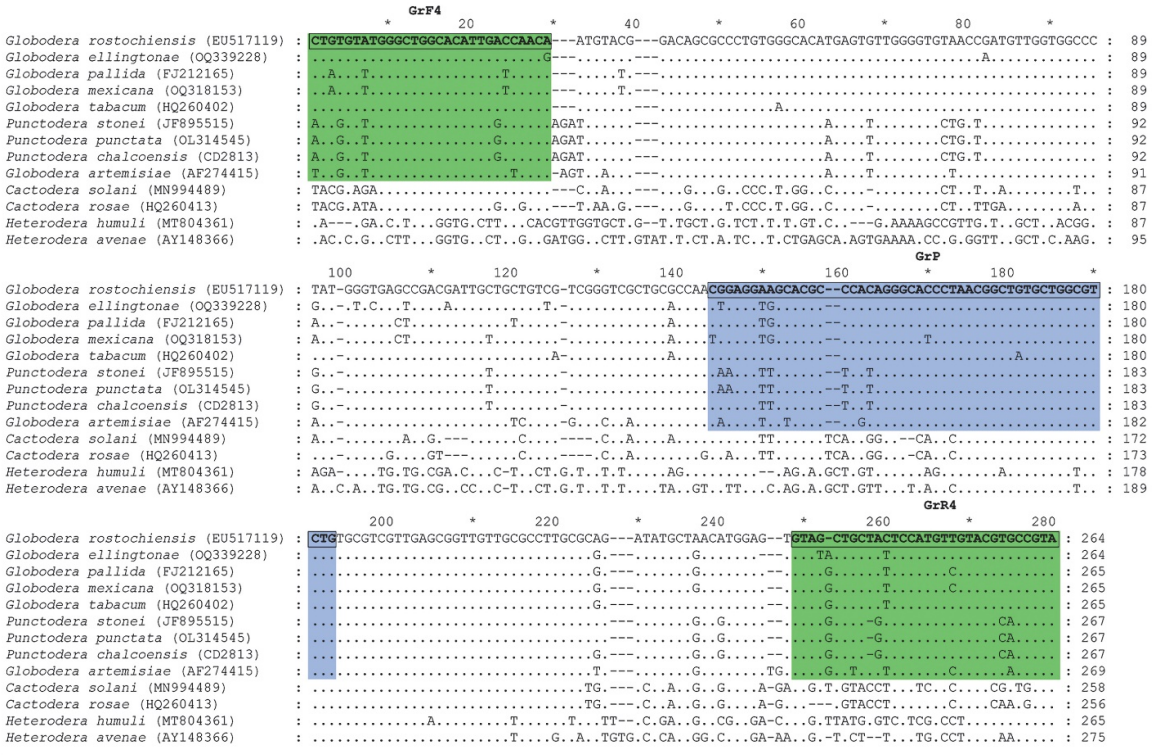

Fig. 2. The fragment of sequence alignment of the ITS rRNA gene for several cyst nematodes with the positions of RPA-LFA primers (GrF4 and GrR4, in green) (Wang *et al.*, 2022) and probe (GrP, in blue) (Wang *et al.*, 2022) used in this study. Coloured areas indicated sequences of species that generated a positive reaction in this assay.

using *in silico* analysis, Subbotin *et al.* (2011) noticed that the *G. rostochiensis* specific primer PITSr3 proposed by Bulman and Marshall (1997) was well matched with the corresponding sequence regions for the ITS rRNA gene of *G. artemisiae*, *G. millefolii* and *Globodera* sp. from Portugal, as well as a *Globodera* sp. from South Africa, differing only in two or three nucleotides and potentially giving false-positive results with these samples.

In Figure 2, the fragment of sequence alignment of the ITS rRNA gene with positions of specific primers and probe in this assay for several tested cyst nematode species is given. Analysis showed that sequences of GrF4 and GrR4 primers have from one to four, and from two to five mismatches, respectively, with gene sequence fragments of species, which give positive reactions in this assay. For GrP probe, mismatches varied from one to six nucleotides. Only one nucleotide mismatch in the 3' end was found between GrF4 and the corresponding gene fragment for *G. ellingtonae*.

It has been known that complementarity between primers and template is often crucial for DNA amplification. Base mismatches located in the 3'-end region affect target amplification much more than mismatches towards the 5'-end, since 3'-end mismatches can disrupt the nearby polymerase active site (Stadhouders *et al.*, 2010). Stadhouders *et al.* (2010) showed that primer mismatches induces a variety of effects on PCR results, they are strongly dependent on mismatch types, their position and the chemicals used. Some studies on this topic have been published for PCR, whereas no data on mismatch behaviour are available for RPA. We believe that lower reaction temperature during RPA might also lead to less specific DNA hybridisation and may have a significant effect in the occurring of false positive results despite mismatches.

Thus, the *Globodera rostochiensis* RPA-LFA detection assay developed by Wang *et al.* (2022) does not give specific detection of this species. Gene fragments containing sufficient nucleotide sequence polymorphism should be selected as diagnostics targets for RPA to obtain a reliable detection of *G. rostochiensis*.

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С.А. Субботин. О надежности рекомбиназной полимеразной амплификации, интегрированной с анализом латерального потока с использованием праймеров и пробы гена ITS рРНК как нового метода обнаружения золотистой картофельной нематоды *Globodera rostochiensis*.

Резюме. Рекомбиназная полимеразная амплификация (РПА) представляет собой метод изотермической амплификации нуклеиновых кислот *in vitro*, который был введен в качестве новой молекулярной технологии для простой, надежной, быстрой и малозатратной диагностики нематод и других вредителей. Недавно РПА, интегрированная с анализом латерального потока (ЛП) с использованием праймеров и пробы гена ITS рРНК, была предложена Wang *et al.* (2022) в качестве нового метода обнаружения *Globodera rostochiensis*. Надежность любого диагностического метода должна быть проверена на широком диапазоне популяций разных видов из разных географических мест, а также на родственных таксонах, чтобы избежать ошибочной идентификации с ложноположительными результатами. В нашем исследовании для подтверждения аналитической специфичности упомянутого выше метода было использовано в общей сложности 24 изолята 13 видов цистообразующих нематод. Результаты испытаний показали, что праймеры и проба гена ITS рРНК не дают с помощью РПА-ЛП специфического определения для золотистой картофельной нематоды и дают положительные реакции не только с *G. rostochiensis*, но и с другими глободерами, паразитирующими на пасленовых растениях, *G. artemisiae* и представителями рода *Punctodera*, паразитирующими на злаках.