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ORIGINAL ARTICLE

Development of a one-step multiplex reverse transcription-polymerase chain reaction and Luminex xTAG assay for the simultaneous detection of yellowing viruses infecting sugar beet

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Abstract

Yellowing viruses are an increasing threat to sugar beet cultivation, due to limitations on insecticide usage and climate change. Virus detection, monitoring and resistance breeding are key to secure high sugar beet yields in the future. For this research, a one-step multiplex reverse transcription (mRT)-PCR method was designed to detect simultaneously beet mild yellowing virus, beet chlorosis virus, beet yellows virus (BYV), beet mosaic virus and turnip yellows virus. The addition of Luminex xTAG array technology was used as a follow-up method to increase assay specificity. The one-step mRT-PCR was evaluated on 22 field samples with single and mixed virus infections. The xTAG assay works as expected both in a simplex and multiplex setting, except that BYV detection needs optimization in the multiplex setting. In the future, the Luminex xTAG assay would be an excellent method for the detection of beet yellowing viruses due to its high specificity and the potential to increase the number of targets.

KEYWORDS
beet yellowing viruses, Luminex assay, methods, multiplex PCR, sugar beet

1 | INTRODUCTION

Virus yellows (VY) disease in sugar beet (*Beta vulgaris*) is caused by aphid-transmitted viruses and can result in yield decreases of up to 47% (Smith & Hallsworth, 1990). The three most important yellowing virus species in sugar beet in Europe are beet mild yellowing virus (BMYV) and beet chlorosis virus (BChV) in the genus *Polerovirus* and beet yellows virus (BYV) in the genus *Closterovirus* (Hossain et al., 2021). In addition, beet western yellows virus (BWYV; genus *Polerovirus*) and beet mosaic virus (BtMV; genus *Potyvirus*) are also known to infect sugar beet causing similar yellowing symptoms. BWYV is not known to occur in the Netherlands, and its detection has only once been described in Europe (Lotos et al., 2014). Turnip yellows virus (TuYV; genus *Polerovirus*) has been classified as a non-beet infecting polerovirus, closely resembling BWYV (Stevens et al., 2005), but recently it has been sporadically found in sugar beet. Newbert (2016) described a TuYV isolate able to infect sugar beet in the UK, and in 2017 a sugar beet-infecting TuYV isolate was detected in Australia (Filardo et al., 2021). Puthanveed et al. (2023) described mixed infections of TuYV with other beet-infecting viruses in sugar beet in Sweden and multiple isolates were detected in recent years in the Netherlands (Schop et al., 2022). As TuYV could be an upcoming viral disease in sugar beet and its symptoms appear to be

similar to other beet-yellowing viruses, timely and reliable detection of this virus as well as the other known yellowing viruses is important for their control.

The major vector species of yellowing viruses in sugar beet is *Myzus persicae*. Other aphid species, such as *Macrosiphum euphorbiae*, have also been described to transmit yellowing viruses (Kozłowska-Makulska et al., 2009; Schliephake et al., 2000). However, their importance in the epidemiology of VY disease is unclear. Yield reduction by virus infection depends on the combination of the virus species, virus strain, sugar beet cultivar and time of infection (Qi et al., 2004; Smith & Hallsworth, 1990; Smith & Hinckes, 1987; Stevens et al., 2004). Smith and Hallsworth (1990) found the highest yield reduction was in fields infected by BYV (47%) compared to 29% for BMYV. A later study performed by Stevens et al. (2004) found a yield reduction up to 27% and 24% caused by BMYV and BChV, respectively. Hossain et al. (2021) observed yield reductions up to 23%, 24% and 10% for BMYV, BChV and BYV, respectively, when inoculation at low density was used to mimic natural infections. Since 1991, viral spread was successfully controlled by the use of neonicotinoid insecticides (Dewar & Qi, 2021). However, since December 2018, the outdoor use of the neonicotinoids clothianidin, thiamethoxam and imidacloprid in the European Union has been restricted (EU Regulation 485/2013), which, in combination with the limitations on suitable chemical substitutes, caused an increase in VY disease in sugar beets (Dewar & Qi, 2021; Laurent et al., 2023).

In the Netherlands, VY infections in beet fields increased from less than 1% before 2019 up to 24%–83% in different regions of the Netherlands in 2020 (Cosun Beet Company, 2021). In 2020, the relative abundance of BMYV, BChV and BYV in VY-positive samples collected in the Netherlands was 51%, 20% and 75%, respectively (Institute of Sugar Beet Research (IRS), 2018, 2020). TuYV, BWYV and BtMV are considered to be absent or rare in the Netherlands and are therefore not monitored at the moment (IRS, 2018, 2020). However, BtMV was detected in 2017 in 24% of samples with yellowing virus symptoms in the UK and 6% of the samples in France (Hossain et al., 2021). Due to climate change and the absence of adequate aphid control measures, it is to be expected that the prevalence of yellowing viruses will increase further in the future (Dewar & Qi, 2021). Therefore, additional testing for TuYV and BtMV seems warranted, as both of these viruses have been detected in recent years in Europe. This, in combination with the large increase in virus incidence of BMYV, BChV and BYV, requires easy, fast and reliable detection of all five yellowing viruses observed in Europe.

Currently, diagnostic tools for the simultaneous detection of beet-infecting yellowing viruses are limited. Testing of BYV in sugar beet can be done by immunocapture reverse transcription-polymerase chain reaction (RT-PCR) (Kundu & Ryšánek, 2004) and simultaneous detection of BMYV and BChV by immunocapture RT-PCR has been reported by Viganó and Stevens (2007). Detection of the three poleroviruses BMYV, BWYV and BChV with RT-PCR was described by Weber et al. (2000). Generic primers for the detection of poleroviruses have been developed by Lotos et al. (2014). In addition, VY virus detection (partly based on the methods described above) was performed by Mahillon et al. (2022), including a new RT-quantitative PCR (RT-qPCR) assay for determining BYV and BChV titres in sugar beet. In addition, a TuYV detection method has been described by Zhang et al. (2016).

However, none of these methods are currently used in the Netherlands for the detection of yellowing viruses. Instead, a two-step RT-PCR method is employed whereby RNA extraction is followed by cDNA synthesis with random primers. Then, cDNA samples are tested for the presence of a polerovirus and BYV in separate PCRs employing generic polerovirus primers and BYV-specific primers. If positive for the presence of a polerovirus, further tests are performed with specific primers for either BMYV or BChV in separate PCRs. As this is a time-consuming and expensive process, which will only increase as more yellowing viruses need to be added, we sought to develop a one-step multiplex RT-PCR (mRT-PCR) as an alternative, in order to test simultaneously for the five yellowing viruses that occur in the Netherlands. To increase specificity of the mRT-PCR, the assay was expanded by the use of target-specific primer extension (TSPE) and Luminex xTAG array technology (Tetyuk et al., 2013; van der Vlugt et al., 2015).

With the Luminex xTAG array technology, an initial RT-PCR with virus-specific primers is followed by TSPE. In TSPE, a primer complementary to a region of the PCR amplicon is used in a linear amplification reaction, during which biotinylated dCTPs are incorporated. Each primer has a specific TAG sequence at its 5′-end that is complementary to an anti-TAG sequence covalently linked to a Luminex bead. Following successful bead hybridization, the biotinylated dCTPs bind a fluorescent streptavidin, R-phycoerythrin conjugate (SA-PE). The fluorescent signal together with the specific bead address is measured by a Luminex MagPix machine. The method has been used successfully for multiplex identification of a wide range of target species, including viruses, viroids, oomycetes and bacteria (Ceyssens et al., 2016; Kostov et al., 2016; Navidad et al., 2013; van Brunschot et al., 2014).

2 | MATERIALS AND METHODS

2.1 | Leaf material

Sugar beet leaves with disease symptoms are sent yearly by Dutch farmers via crop advisors to IRS for diagnostic testing, including testing for fungi, bacteria and viruses. The date of receipt, location of sampling and the diagnostic results are stored. Plant material with suspected viral symptoms is checked for virus infection by RT-PCRs, and the RNA is subsequently stored at −80°C.

2.2 | RNA extraction and the two-step PCR protocol

Plant sap was collected by squeezing the tip of a sugar beet leaf with a custom-made hand lever press. Subsequently, 50 μL of plant sap was

diluted in 450 µL conjugate buffer (8 g NaCl, 2.9 g $Na_2HPO_4.12H_2O$, 0.2 g KH_2PO_4, 0.2 g KCl, 0.5 mL Tween 20, 20 g polyvinyl pyrrolidone (K10-K40) and 2 g bovine serum albumin in 1 L distilled water, pH 7.4) and stored at −20°C until RNA extraction. Total RNA was extracted using the QiaAmp Viral RNA Mini kit (Qiagen) according to the manufacturers' protocol and eluted in 50 µL AVE elution buffer. The purity was determined by measuring the ratio of absorbance at 260 nm (A_{260}) to A_{280}. RNA concentration was measured with a NanoDrop One spectrophotometer (Thermo Scientific).

For the detection of yellowing viruses, RNA extraction was followed by a standard two-step PCR protocol at IRS. For this, cDNA synthesis is performed with the RevertAid RT Reverse Transcription Kit from ThermoFisher Scientific according to the manufacturers' protocol with a final reaction volume of 10 µL. Subsequently, cDNA samples are tested for the presence of a polerovirus and BYV in separate PCRs employing generic polerovirus primers and BYV-specific primers using the Phusion Flash High-Fidelity Master Mix from ThermoFisher Scientific. If tested positive for the presence of a polerovirus, further tests are performed with specific primers for either BMYV or BChV in a separate PCR. BMYV, BChV and BYV primers were designed by Menzel et al. (2002) (Leibniz Institute DSMZ, Braunschweig, Germany; Table 1), and the generic poleroprimers were obtained from Hossain et al. (2021). RT-PCR was carried out in a T100 thermal cycler (Bio-Rad). Annealing temperature differed per target and is given in Table 1. The protocol was as follows: 10 s at 98°C for initial denaturation; 30 cycles of denaturation of 1 s at 98°C, annealing for 5 s at X°C (Table 1) and extension for 15 s at 72°C; and a final step of 1 min at 72°C. PCR amplicons were checked by gel electrophoresis. For this, 5 µL of PCR product was mixed with 1 µL of TriTrack DNA loading dye (6×, Thermo Scientific) and loaded on a 1.5% agarose gel in 0.5× TBE buffer (40 mM Tris-borate [pH 8.0], 1 mM EDTA). After gel electrophoresis, the DNA was stained in a bath containing 0.5 µg/mL ethidium bromide for 20 min, after which the gel was washed in a water bath. Pictures were taken with a Molecular Imager Gel Doc XR+ System and analysed with Image Lab Software (Bio-Rad).

2.3 | One-step mRT-PCR and Luminex xTAG assay

2.3.1 | PCR protocol

For the one-step mRT-PCR, the OneStep RT-PCR Kit (Qiagen) was used. For this, specific primers were designed in the ORF1 regions of the viruses, as described in the section below; their sequences are given in Table 2. *NAD5* primers (Menzel et al., 2002) were used in a separate reaction as a control to prove RNA was present and of good enough quality to be amplified (forward: 5′-GATGCTTCTTGGGGCTTCTTGTT-3′; reverse: 5′-CTCCAGTCAC CAACATTGGCATAA-3′).

For each reaction, 1 µL of template RNA was added to 0.5 µL of each primer (10 in total; each with a concentration of 10 mM), 1 µL enzyme mix, 5 µL buffer (5×), 1 µL dNTP mix (10 mM) and 12 µL water to obtain a total reaction mix of 25 µL. RT-PCR was carried out in a T100 thermal cycler (Bio-Rad) using the following conditions: 30 min at 50°C for reverse transcription of the RNA template, followed by 15 min at 95°C for initial denaturation and subsequently, 37 cycles of 30 s at 94°C, 30 s at 56°C and 1 min at 72°C; and a final step of 10 min at 72°C. PCR amplicons were checked by gel electrophoresis.

2.3.2 | Outer primer design

All available complete genomes of BMYV, BChV, BYV, BtMV and TuYV from NCBI were aligned to the RefSeq sequences of the corresponding virus (NC_003491, NC_002766, NC_001598, NC_005304 and NC_003743, respectively). A list of the available genomes used can be found in Data S1. Primers were designed with the function Design Primers in CLC Genomics Workbench v. 20.0 (http://digitalinsights.qiagen.com/). Primers for detection of specific poleroviruses were designed to be at the 5′ end of the genome, as this region shows lowest homology between different poleroviruses (Weber et al., 2000). Amplicons were designed to be of different sizes per virus, making it easy to distinguish between them on an agarose gel.

TABLE 1 Primer sequences for the detection of yellowing viruses in beet.

Virus species	Product size (bp)	Direction	Sequence (5′–3′)	Annealing temp. (°C)
Polero (generic)	482	Forward	CAGCCAGTGGTTGTGGTCC	69
		Reverse	GCTATCGATGAAGAACCATTGCCTT	
BYV	800	Forward	GTTAACACAGTTACTAAGGTTCCA	61.4
		Reverse	TGGAGGTATACCAAAGGAAGTTCA	
BMYV	800	Forward	ACTGCATTCGTTCTCTTCTCG	68
		Reverse	CATACCGCGAGCTTCACCAAA	
BChV	600	Forward	GTGACGAGCGAAAGACACTTG	68
		Reverse	CGCTTAAGGCCATCAATGAGG	

Note: BYV, BMYV and BChV primers were designed by Menzel et al. (2002) (Leibniz Institute DSMZ, Braunschweig, Germany, unpublished). The generic poleroprimer set was obtained from the publication of Hossain et al. (2021).

Abbreviations: BChV, beet chlorosis virus; BMYV, beet mild yellowing virus; BYV, beet yellows virus.

TABLE 2 Primers for simultaneous detection of BMYV, BChV, BYV, BtMV and TuYV in a one-step multiplex reverse transcription-PCR (mRT-PCR).

Virus species	Target	Product size (bp)	Direction	Sequence (5′–3′)
BMYV	ORF1	632	Forward	GTATTCGTTCTCTTCTCGCT
			Reverse	CGGGCTTTGTTTGATGGTA
BChV	ORF1	297	Forward	GACACCAAGTTGAACAGT
			Reverse	CATTGATGCTAGTTGTGGC
BYV	ORF1	491	Forward	CGAGGCGGCAAATTAAGT
			Reverse	GTTGTCTTTGTGAATGCTG
BtMV	P3[a]	834	Forward	GGAATGGCGAGGTTTAAG
			Reverse	CTTGTCCCACCACTTCTC
TuYV	ORF1	142	Forward	TGGAGCTCGCTAATCTTG
			Reverse	GGCAATCCTCCAAAAAGAA

Abbreviations: BChV, beet chlorosis virus; BMYV, beet mild yellowing virus; BYV, beet yellows virus; BtMV, beet mosaic virus; TuYV, turnip yellows virus.

[a]BtMV contains only one open reading frame (ORF), which is translated into a polyprotein and subsequently cleaved.

TABLE 3 Target-specific primer extension (TSPE) primers for the Luminex xTAG Array Technology.

Virus species	Sequence (5′–3′)	Luminex xTAG bead number
BMYV	***CAATTTACATTTCACTTTCTTATC***-GTATGGATTTTTGGCGGT	MTAG-A051
BChV	***TCATCACTTTCTTTACTTTACATT***-TCAGATTTTCCCTTACCACC	MTAG-A034
BYV	***CTATCATTTATCTCTTTCTCAATT***-TTTTACAGTTTGCCGCGTT	MTAG-A072
BtMV	***TTAACAACTTATACAAACACAAAC***-CAAATTCGTCATATCGCCCA	MTAG-A053
TuYV	***CATCTTCATATCAATTCTCTTATT***-GTCTACRCCGAAATGTTTG	MTAG-A035

Note: The primer part in bold and italics is the region complementary to the anti-TAG hybridization sequence on the Luminex bead. Primers were designed for the detection of BMYV, BChV, BYV, BtMV and TuYV.

Abbreviations: BChV, beet chlorosis virus; BMYV, beet mild yellowing virus; BYV, beet yellows virus; BtMV, beet mosaic virus; TuYV, turnip yellows virus.

2.3.3 | TSPE primer design

Virus-specific TSPE primers were designed to be located in between the corresponding forward and reverse primer for each virus amplicon. The six bases at the 3′ end of the primer sequences were tested in silico for absence of nonspecific binding with any of the other virus sequences. Virus-specific TSPE primer sequences were elongated at the 5′ end with a specific TAG sequence from which guanine nucleotides are excluded. The TAG sequence is complementary to an anti-TAG sequence coupled to a specific colour-coded Luminex bead. Primer sequences are given in Table 3.

2.3.4 | xTAG array technology protocol

After running the one-step mRT-PCR, amplicons were purified with 1.2 μm filter plates (Merck) filled with Sephadex (G-50; GE Healthcare) according to the manufacturers' protocol. Subsequently, TSPE amplification was performed in a 20 μL solution by adding 2 μL of PCR buffer (10×) to 0.5 μL $MgCl_2$ (50 mM), 0.25 μL biotin-dCTP (400 μM), 1 μL d(ATG)TPs (100 μM), 0.25 μL of each TSPE primer (five primers in total) (2 μM), 0.15 μL Tsp polymerase (5 U/μL), 9.85 μL Milli-Q water and 5 μL purified mRT-PCR amplicons. The PCR programme was 96°C for 2 min for initial denaturation and 35 cycles of 20 s at 94°C, 30 s at 55°C and 30 s at 72°C. For the hybridization of the TSPE amplicons to the anti-TAG sequences linked to a specific coloured, paramagnetic bead, 25 μL of hybridization buffer ([2×] 0.4 M NaCl, 0.2 M Tris, 0.16% Triton X-100, pH 8.0 filter sterilized stored at 4°C; Angeloni et al., 2022) was added to 0.5 μL of each xTAG solution (anti-TAG with coloured bead), 17.5 μL of Milli-Q water and 5 μL of TSPE product to obtain a solution of 50 μL. The programme for hybridization was 2 min at 96°C and 30 min at 37°C. Subsequently, the hybridized samples were washed and streptavidin, R-phycoerythrin conjugate (SA-PE) solution (Moss Inc.) was added, which is a biotin-binding protein covalently attached to a fluorescent label. For washing, 50 μL of hybridized sample was pipetted into a Greiner flat bottom 96-well polystyrene cell culture microplate (655180) and put on a Luminex magnet for 1 min to bind the magnetic beads to the bottom of the wells. The plate was flicked dry, 100 μL of hybridization buffer (1×) was added and the plate removed from the magnet. After 1 min, the plate was replaced on the magnet and flicked dry again. This washing step was repeated once. Finally, 100 μL of SA-PE solution (1000× diluted in hybridization buffer) was added to the hybridized samples and incubated for 15 min at room temperature at 600 rpm in the dark, after which the plate was placed on the magnet and washed again. Finally, 100 μL of

hybridization buffer was added and the plate was put in the Luminex MagPix for measurement of fluorescence. Threshold values were calculated per bead address by calculating the average and standard deviation of the median fluorescence intensity (MFI) values of the negative control and the samples that were previously shown to be negative for the corresponding virus. Subsequently, the threshold value was calculated by using the following formula: threshold value = (average + [3 × *SD*]) × 1.1.

3 | RESULTS

3.1 | mRT-PCR and analytical specificity test

To detect simultaneously the five yellowing viruses BMYV, BChV, BYV, BtMV and TuYV in sugar beet, a one-step mRT-PCR was developed by designing primers specific to the ORF1 regions of these five viruses. To exclude possible primer cross-reactivity, RNA samples from single viral infections (confirmed with the two-step PCR method) were used to test the one-step multiplex primers for specificity. For this, combinations of each of the five viruses with each of the five primer pairs were made and the identity of any amplicon was confirmed by sequencing. Figure 1a shows the specificity of the primers as almost no cross-reactivity with RNA of the other viruses was observed. Subsequently, simultaneous detection of BMYV, BChV, BYV, BtMV and TuYV was performed by mixing RNA templates and primer pairs in single mRT-PCRs. Figure 1b shows no cross reactions were observed when primer pairs were combined and tested on individual RNAs and all five viruses were detected in the mRT-PCR containing a mix of their RNAs. The sequences of the individual amplicons were confirmed by Sanger sequencing. The very faint bands in Figure 1a that can be observed for BChV and BMYV when using TuYV primers and BChV when using BYV primers are only present in a simplex setting and were therefore not investigated further and no more steps were taken to try to remove them.

3.2 | Diagnostic performance on field samples

To investigate reliability of the primers for simultaneous detection of BMYV, BChV, BYV, BtMV and TuYV, they were tested on field samples that had been proven to have single or mixed infections (with the two-step PCR method). For this, 22 field samples collected between 2019 and 2021 from 19 locations dispersed over the Netherlands were used (Data S2). Figure 2 shows the results of the mRT-PCR on these field samples containing single or mixed infections in different combinations of the five yellowing viruses. In Lane 23, the positive control consisting of a mixture of BMYV, BChV, BYV, BtMV and TuYV RNA shows accurate amplification of all five virus fragments. Primers did not produce unspecific amplicons in the negative controls in Lanes 22 (virus-free sugar beet), 24 and 25 (water). In total, five BMYV, four BChV, four BYV and one TuYV amplicons were sent for Sanger sequencing and all sequences were similar to their corresponding virus (Data S3). Most of the results were similar to those obtained with the two-step method previously used at IRS for the detection of yellowing viruses, except for samples in Lanes 21 and 22, in which a faint BChV band can be observed that was not detected with the two-step PCR method. In another experiment, a set of 20 samples positive for BMYV, BChV and/or BYV was tested with the mRT-PCR method and compared to the two-step PCR method. The mRT-PCR method detected three BMYV, three BChV and one BYV infection in the samples that were previously found negative for these viruses (Data S4). Sanger sequencing confirmed the correct virus identity of these amplicons. Thus, the sensitivity of the mRT-PCR method was higher than the conventional two-step RT-PCR.

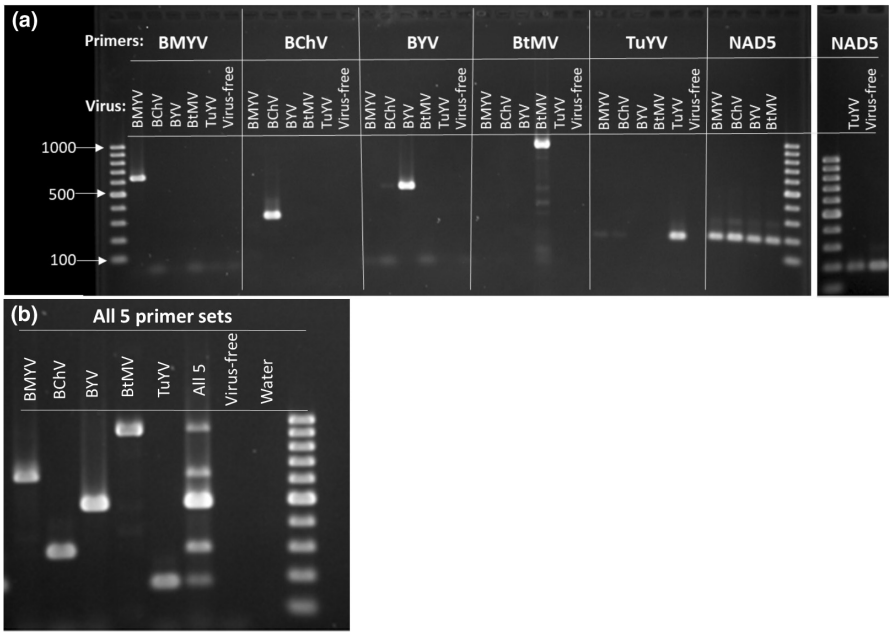

FIGURE 1 Results of the multiplex reverse transcription-PCR designed for detection of beet mild yellowing virus (BMYV; 632 bp), beet chlorosis virus (BChV; 296), beet yellows virus (BYV; 491 bp), beet mosaic virus (BtMV; 834 bp) and turnip yellows virus (TuYV; 142 bp). (a) Single primer pairs tested on RNA from leaf material infected with a single virus. (b) Mixture of all five primer pairs tested on RNA from a single virus or a mixture of viral RNAs.

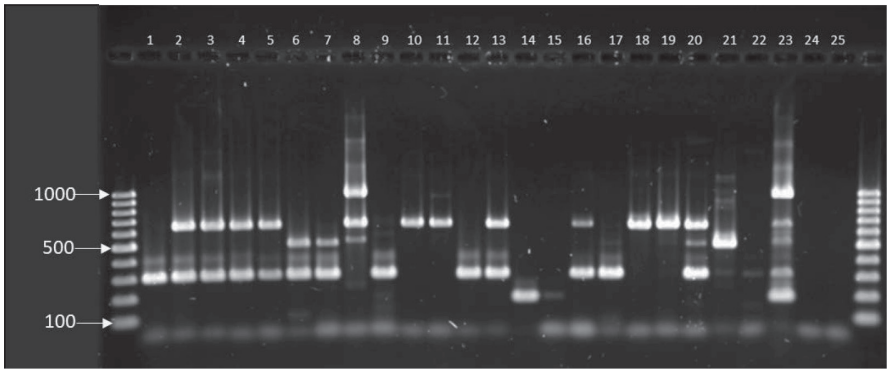

FIGURE 2 The results of one-step multiplex reverse transcription-PCR with 22 RNA samples from virus-infected leaf material of beet mild yellowing virus (BMYV; 632 bp), beet chlorosis virus (BChV; 296 bp), beet yellows virus (BYV; 491 bp), beet mosaic virus (BtMV; 834 bp) and turnip yellows virus (TuYV; 142 bp).

	Viral RNA
1	BChV
2	BChV + BMYV
3	BChV + BMYV
4	BChV + BMYV
5	BChV + BMYV
6	BYV + BChV
7	BYV + BChV
8	BMYV + BYV + BtMV
9	BChV

	Viral RNA
10	BMYV
11	BMYV
12	BChV
13	BChV + BMYV
14	TuYV
15	TuYV (low infection)
16	BChV + BMYV
17	BChV
18	BMYV

	Viral RNA
19	BMYV
20	BMYV + BChV + BYV
21	BYV
22	No virus
23	BMYV + BChV + BYV + BtMV + TuYV
24	Water
25	Water

TABLE 4 Median fluorescence intensity values from a Luminex xTAG array of RNA of single viruses from beet plant material infected with BMYV, BChV, BYV, BtMV or TuYV.

Virus	BMYV #51	BChV #34	BYV #72	BtMV #53	TuYV #35	PCR no.
NC	64	103	63	95	93	30
BYV	45	79	**281**	75	82	9
BYV	47	83	**1510**	85	79	10
BYV	51	81	**1526**	85	83	11
BMYV	**5129**	89	70	87	85	1
BMYV	**5209**	92	66	96	90	2
BMYV	**5751**	85	59	77	83	3
TuYV	49	83	59	82	**1343**	31
BtMV	63	89	62	**4076**	83	25
BtMV	67	97	75	**4615**	104	26
BChV	338	**5758**	62	84	90	17
BChV	426	**6864**	75	95	100	18
BChV	393	**7091**	62	92	89	19
Average	154	88	65	87	88	–
SD	161.3	7.4	6.0	7.3	7.6	–
Threshold value	701.9	121.4	91.4	119.3	122.3	–

Note: The bead address numbers are given in the top row. The last column refers to the sample number from the gel electrophoresis picture in Data S4. For the negative control, water was added instead of RNA during outer primer amplification. The values above the threshold value are in bold and underlined; these samples are considered positive for the respective virus.

Abbreviations: BChV, beet chlorosis virus; BMYV, beet mild yellowing virus; BYV, beet yellows virus; BtMV, beet mosaic virus; TuYV, turnip yellows virus.

3.3 | TSPE and Luminex xTAG array technology

To increase specificity of the mRT-PCR and have the potential to increase the maximum number of targets, the method was expanded with the TSPE and Luminex xTAG system, which allows testing for multiple targets simultaneously.

Initial amplification was performed using the 'outer primers' according to the one-step mRT-PCR protocol. Results of the gel electrophoreses can be found in Data S5 and S6. Subsequently, unidirectional TSPE reactions using TSPE primers were performed, with amplicons detected by hybridization to anti-TAG sequences covalently bound to beads and addition of phycoerithryn-labelled streptavidin. Individual bead counts and associated fluorescence levels were measured in a Luminex MagPix.

Single infections with BMYV, BChV, BYV, BtMV and TuYV were used to investigate whether the TSPE primers were able to bind to

the corresponding viral amplicon and if the TAG and its complementary anti-TAG sequences were able to bind and thereby give an MFI signal in the Luminex. First, only the outer primers and RNA from individual viruses were used to test TSPE specificity (Table 4). Subsequently, viral RNA obtained from field samples was mixed to obtain samples with a combination of RNAs from different yellowing viruses. As an additional control, instead of mixing the viral RNAs before mRT-PCR, viral amplicons of each virus from outer primer amplification of single infections were mixed and used subsequently for TSPE amplification and xTAG Array Technology (Table 5).

MFI levels are shown in Tables 4 and 5. All viral amplicons could be detected by the Luminex xTAG Array Technology when the samples consisted of either single virus infections (Table 4) or mixed infections (Table 5). MFI values of bead BMYV #51 are relatively high in samples where only BChV is present, which results in a high threshold value. The high MFI values could be the effect of low unspecific binding of the BMYV TSPE primer to BChV amplicons. As there were no BMYV outer primers used during this assay of the BChV-infected samples, it is unlikely it is the result of a low BMYV viral infection. Although threshold values are relatively high, the MFI values of BMYV-infected samples are over 10 times higher than the BMYV uninfected samples and therefore the primers and corresponding Luminex signals are still regarded as specific. Thus, the TSPE and Luminex xTAG for VY virus detection can be used both as a simplex or multiplex assay. The results given in Table 4 show that the Luminex xTAG assays worked as expected on samples infected with a single virus where multiple beads and TSPE primers were used in one reaction mixture.

In mixed infections (Table 5), the method also detected all viruses correctly. Again, relatively high threshold values were observed for BMYV bead #51. However, this did not affect the results, as, once more, the difference in MFI values between positive and negative BMYV samples was large. An apparent MFI value of 998 for BYV bead #72 was observed for an RNA mixture with BMYV and BChV (Table 5). However, the RNAs used to make this mixture were also assessed in the first assay (Table 4) and did not result in a BYV-positive signal. In addition, with gel electrophoresis no band (491 bp) corresponded to the expected size of a BYV amplicon in any of these samples (see Data S6, Lane 8), suggesting this high MFI value was caused by experimental error.

4 | DISCUSSION

With the increase of VY in sugar beet over the last 3 years, there is a growing need for a broader and more reliable virus detection method, as breeders, farmers and legislators need to know which viruses are prevalent. We have designed a method for simultaneous detection of BMYV, BChV, BYV, BtMV and TuYV, of which BMYV, BChV, BYV and BtMV are common throughout Europe (Hossain et al., 2021) and TuYV may be a virus of concern for the future. Although BtMV is not yet very common in the Netherlands, it is also a nonpersistently aphid-transmitted virus with a wide host range; its incidence is expected to increase in the future due to the increase in aphid and virus prevalence in general, the absence of effective insecticide control and climate change (Dewar & Qi, 2021). Since

TABLE 5 Median fluorescence intensity values from a Luminex xTAG array of RNA of single or mixed viruses from beet plant material infected with BMYV, BChV, BYV, BtMV or TuYV.

Virus	BMYV #51	BChV #34	BYV #72	BtMV #53	TuYV #35	PCR no.
NC	97	130	116	127	122	17
NC 2	154	155	163	166	175	28
BChV	394	**5337**	140	179	172	19
BMYV + BYV + BChV	**2424**	**5965**	**528**	186	187	2
BMYV + BYV + BChV	**1273**	**5350**	**422**	159	154	3
BMYV + BYV	**2108**	166	**459**	172	189	5
BMYV + BYV	**1469**	160	**339**	164	152	6
BMYV + BChV	**4020**	**5987**	**998**[a]	154	155	8
BMYV + BYV + BChV + BtMV + TuYV	**1133**	**4082**	**703**	**2097**	**326**	16
BMYV + BYV + BChV + BtMV + TuYV[b]	**2784**	**6955**	**477**	**3783**	**599**	–
Average	215	153	140	163	163	–
SD	157	16	24	18	22	–
Threshold value	755.5	220.2	231.8	239.5	252.0	–

Note: The bead address numbers are given in the top row. The last column refers to the sample number from the gel electrophoresis picture in Data S5. For the negative control, water was added instead of RNA during outer primer amplification. The values above the threshold value are in bold and underlined; these samples are considered positive for the respective virus.

Abbreviations: BChV, beet chlorosis virus; BMYV, beet mild yellowing virus; BYV, beet yellows virus; BtMV, beet mosaic virus; TuYV, turnip yellows virus.

[a]This value is unexpectedly high and is likely to be the result of an experimental mistake.

[b]Positive control where viral amplicons were mixed after outer primer amplification instead of mixing viral RNA before outer primer amplification.

2020, TuYV has been sporadically found in Dutch sugar beet fields with an as yet unknown risk status; therefore, it might be important in the coming years and should be monitored.

The one-step mRT-PCR method presented here was evaluated on a set of virus-infected leaves collected from Dutch sugar beet fields and was shown to detect all virus infections correctly. It appeared to be even more sensitive than the two-step PCR method, because one-step mRT-PCR detected low infections of BMYV, BChV and BYV (confirmed by sequencing of amplicons) that had not been detected by the two-step procedure.

In our investigation, we used conventional mRT-PCR with gel electrophoresis in combination with Luminex xTAG Array Technology rather than quantitative real-time PCR, such as TaqMan and SYBR Green, as it enabled the detection of many more targets in one assay. With gel electrophoresis, samples are regarded as positive based solely on the size of an amplicon, which carries the risk of false positives and may require sequence confirmation of the amplicons. The additional use of the Luminex xTAG Array method is not only truly multiplex and very sensitive, but also includes an extra level of specificity through the use of amplicon-specific primers (van der Vlugt et al., 2015). This method was successfully employed previously for detection of nine pospiviroid species in one assay (van Brunschot et al., 2014) and for identification of 48 members of the genus *Phytophthora* up to clade, subclade or species level (Kostov et al., 2016). The method is semiquantitative and due to its potential to detect large numbers of targets in a single assay, additional specific or generic primers can easily be added for new viruses, mutants or recombinants of known viruses.

Here, TSPE primers were designed for all five yellowing viruses present in the Netherlands and successfully used to detect the five viruses when tested on single and mixed VY infected field samples. BMYV TSPE primers appear to give a low background reaction in the presence of BChV amplicons, but this does not affect the specificity of the Luminex as MFI values in actual BMYV-infected samples are over three times as high. It is unclear what caused the high MFI value for BYV in a sample with only BMYV and BChV infection, and it was not detected in the assay where the same BMYV and BChV isolates were tested separately. We assume this high BYV MFI signal was nonspecific and caused by an experimental error as no BYV amplicon was formed with this sample in the first RT-PCR amplification step. However, further optimization of the multiplex setting is recommended regarding BYV detection. Nevertheless, we have shown a proof of concept as well as the multiplex potential of this Luminex assay, which, apart from the exceptions noted, detected all viruses from both single and mixed virus-infected leaf material.

The epidemiology of VY disease, and its monitoring in the field, is important for breeders and farmers to enable the selection of suitable virus-resistant cultivars for growth in specific locations. The information can also be used to regulate insecticide application at a local scale. The mRT-PCR is cheap and fast as it detects all targets of interest in a single assay. The benefit of the Luminex xTAG assay is that it can easily be expanded to include additional viruses, for example BWYV. Additional genus-specific primers for well-conserved regions of viral genera, such as poleroviruses, could be added to even increase specificity further, limiting the risk of overlooking mutants and allowing detection of so far unknown viruses. Also, an additional primer set binding to a housekeeping gene of plants, such as *NAD5*, could be added as a positive control for the RNA extraction and Luminex xTAG procedures. In a further expansion of this method, the Luminex xTAG assay could be optimized for detecting viruses in aphids. A small laboratory-based pilot study showed that in most cases the viruses could be detected in single *M. persicae* aphids (results not shown). In this way, aphids belonging to different species collected in traps could be easily checked for the presence of viruses, which would be useful to discover the role and possible importance of other aphid species in virus epidemiology. Thus, we have shown that the combination of a mRT-PCR with the Luminex xTAG array technology is a sensitive method to screen multiple samples for different viruses in a single assay.

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

Data are available on request from the authors.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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