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ORIGINAL ARTICLE

Survey on phytoplasmas associated with grapevine yellows in Eastern Georgia, Caucasus region

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Abstract

During field surveys conducted from July to October 2018–2020 in the eastern part of Georgia (Caucasus region), 145 out of 8000 (1.8%) and 147 out of 6600 (2.2%) grapevine plants, respectively, from mother stock/collection fields and commercial vineyards, were found exhibiting typical or suspicious grapevine yellows (GY) symptoms. Most of the symptomatic grapevine plants of Georgian cultivars showed mild symptoms with no berry alterations. Leaf samples from symptomatic plants were analyzed by serological (DAS--ELISA) and molecular (two previously published protocols of TaqMan triplex real-time PCR, here named Set I and Set II) tools for detecting GY-associated phytoplasmas. The presence of GY phytoplasmas was not detected in any examined grapevines by a serological method. GY phytoplasmas were identified in 22.41% and 6.9% symptomatic plants from mother stock and collection vineyards and in 48.3 and 19.0% symptomatic plants from commercial vineyards by Set I and Set II PCRs, respectively. As expected from previous studies reporting the wide presence of bois noir (BN) in Georgian vineyards, '*Candidatus* Phytoplasma solani'(CaPsol) was detected in most phytoplasma-infected plants (47.6%), with the highest infection rate in Chardonnay. Phytoplasmas belonging to taxonomic group 16SrV were detected in 45.6% of the phytoplasma-infected grapevines. To the best of our knowledge, this is the first report of 16SrV phytoplasmas in Georgia and in the Caucasus region. Further molecular typing of 16SrV phytoplasma strains is necessary to determine if such strains are associated with flavescence dorée (FD). The knowledge of typical GY symptoms and the utilization of accurate diagnostic tools are crucial for preventing pathogen spread and producing healthy planting material. Based on the results obtained in this study, the presence of BN and 16SrV phytoplasmas should be monitored in the next years using triplex real-time PCR.

Keywords: bois noir, detection, flavescence dorée, 16SrV

Introduction

Flavescence dorée (FD) and bois noir (BN), two diseases of the grapevine yellows (GY) complex, are associated with "flavescence dorèe" phytoplasmas (FDp) belonging to taxonomic subgroups 16SrV-C and -D (Martini *et al.* 1999), and to '*Candidatus* Phytoplasma solani' (CaPsol) (Quaglino *et al.* 2013), respectively. Symptoms of FD and BN are undistinguishable and include leaf curling and color alterations (yellowing in white varieties and reddening in red varieties), irregular wood maturation of shoots, and berry shrivel.

Both diseases seriously decrease production in grapevine varieties in European countries (Belli *et al.* 2010). FD is the most severe GY disease in Europe due to its highly epidemic spreading. In fact, FDp are vine-to-vine transmitted by the insect vector *Scaphoideus titanus* Ball (Homoptera: Cicadellidae), a vine feeding leafhopper of American origin (Schvester *et al.* 1963). Moreover, recent studies demonstrated that FDp ecology is more complex than the close pathosystem 'grapevine – *S. titanus*'. In detail, wild plants of *Alnus glutinosa*, *Corylus avellana*, and *Calystegia sepium* were found as FDp reservoirs for vector insects such as *Oncopsis alni*, *Dictyophara europaea*, and *Orientus oshidae* capable of incidentally transferring FDp to grapevines in vineyards and creating the conditions for *S. titanus*-related FD epidemics (Casati *et al.* 2017; Malembic-Maher *et al.* 2020). Although FDp is a quarantine pathogen according to the European Community (EEC 77/93) and its control is regulated by binding regulation, new outbreaks have been reported in recent years (Angelini *et al.* 2018). CaPsol, associated with BN, is occasionally transmitted to grapevines by *Hyalesthes obsoletus* Signoret (Homoptera: Cixiidae) (Maixner *et al.* 1994), a polyphagous planthopper living preferentially on nettle (*Urtica dioica* L.), bindweed (*Convolvulus arvensis* L.), and chaste tree (*Vitex agnus-castus* L.) (Langer and Maixner 2004; Kosovac *et al.* 2016). Moreover, recent studies demonstrated that (i) the cixiid *Reptalus panzeri* is a vector of CaPsol in Serbian vineyards (Cvrković *et al.* 2014), (ii) other insects are alternative vectors of CaPsol to grapevines in northern Italy (Quaglino *et al.* 2019), and (iii) several weeds present in the vineyard agroecosystem are involved in CaPsol epidemiology (Kosovac *et al.* 2019; Moussa *et al.* 2019; Quaglino *et al.* 2021). Because of the CaPsol multifaceted ecology, including multiple insect vectors and plant hosts, it is extremely difficult to develop efficient control strategies for BN. According to current data none of the *Vitis* spp. and *Vitis vinifera* L. varieties are resistant to phytoplasma infection (Laimer *et al.* 2009).

Georgia is the oldest vine growing and winemaking country in the world and motherland of several grapevine (*Vitis vinifera* L.) varieties. According to scientific information, wine manufacturing in Georgia began in the early Neolithic period (McGovern *et al.* 2017). More than 500 Georgian grapevine cultivars are characterized by an exclusive genetic variability and are different from that of Europe and Central Asia (Chkhartishvili and Maghradze 2012; Bacilieri *et al.* 2013; Imazio *et al.* 2013). Saperavi (red) and Rkatsiteli (white) are the most cultivated varieties, famous also outside Georgia, and can be found in Eastern Europe and Central Asia. According to recent studies, viruses and phytoplasmas associated with grapevine diseases have been reported in Georgian vineyards. Among viral pathogens, grapevine fanleaf virus (GFLV), grapevine fleck virus (GFkV), and grapevine leafroll-associated virus-1 and -3 (GLRaVs) are present in all Georgian regions (Megrelishvili *et al.* 2016; Elbakidze *et al.* 2021). Among phytoplasmas, up to now, only CaPsol has been identified in association with grapevine yellows throughout the country. Most CaPsol-infected Georgian varieties exhibited moderate and mild symptoms, enabling good production to be maintained (Quaglino *et al.* 2014, 2016).

Due to the different ecology of their associated phytoplasmas, leading to distinct epidemiological patterns, the control strategies of FD and BN are different. As stated above, FD control is mandatory and involves the use of certified planting materials, the removal of infected vines, and the control of *S. titanus* populations. On the other hand, BN control is not regulated by mandatory measures and due to its complex ecology, it is difficult to develop efficient control strategies. If FD and BN, characterized by undistinguishable symptoms, are present in the same viticultural area, reliable molecular methods must be used to specifically identify FDp and BNp in symptomatic grapevines (Angelini *et al.* 2018).

The main goal of this research was to survey by serological and molecular methods the presence of phytoplasmas associated with FD and BN in vineyards located in the eastern part of Georgia, including Kakheti and Kartli regions, for developing a plant sanitary system in the country.

Materials and Methods

Sample collection

During field surveys conducted from July to October 2018 to 2020 in the eastern part of Georgia, including Kakheti (Kondoli, Napareuli, Tsinandali villages) and Kartli (Jigaura and Mukhrani villages) regions, 14,600 grapevines (8,000 from three mother stocks, three nursery collection fields, and 6,600 from six commercial vineyards) were investigated. The grapevine plants belonged to 16 grapevine cultivars including the five most popular and widely used commercial varieties [Chardonnay (1,100 plants), Khikhvi (1,300), Saperavi (1,800), Cabernet sauvignon (1,400), Manavi mtsvane (1,000)]. Samples of leaves with petioles were collected from grapevines exhibiting typical or suspicious symptoms of grapevine yellows (GY) and stored at 4°C for 2–3 days until serological analysis or DNA extraction was carried out.

Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA)

The DAS-ELISA test was performed on extracts of young leaves with petioles using a serological commercial kit (SEDIAG, Switzerland). Leaves and petioles of tested samples were crushed [(w/v) 1 : 5] in extraction buffer (pH 8.2) containing 2% polyvinylpyrrolidone (PVP Mw 24,000), 0.8% NaCl, 2.42% Tris, 0.02% NaN_3, and 0.05% Tween 20. Coating and conjugated polyclonal antibodies for FDp and BNp and controls included in the kit were used following the washing and incubation steps as per the manufacturer's instructions. Samples as well as controls were run in duplicate. Microtiter plates were loaded onto ELX800 Microplate Reader (Bio-Tek Instruments, Winooski, VT, USA) and absorbance value was determined at 405/450 nm. Grapevine samples with optical density three times higher than the mean of negative controls were considered as phytoplasma infected (Cooke *et al.* 2006).

DNA extraction

For each symptomatic grapevine sample, 100 mg of mixed petioles were used for DNA extraction and the rest was stored at –20°C for further use. Total DNA was extracted using PureLink® Plant Total DNA Purification Kit (Invitrogen™, USA) according to the manufacturer's instructions. DNA was eluted in 100 µl PureLink Elution Buffer (E1). The concentration of DNA was measured using Qubit™ 4 Fluorimeter (Invitrogen, USA). Extracted DNAs ranged from 50 ng · µl^{-1} to 100 ng · µl^{-1}.

PCR assays

TaqMan® triplex real-time PCR using TaqMan®MGB™ probes (Applied Biosystems) were used to detect different 16SrV phytoplasma strains, including FD-associated phytoplasmas, and BN phytoplasma from the 16SrXII-A subgroup. Two sets of primers and probes, previously described by Pelletier *et al.* (2009) and Angelini *et al.* (2007), were applied. Probes for 16SrV and BN phytoplasmas were 5'-labelled with FAM™ and VIC™ reporter dye, respectively (Table 1).

Reactions were performed in a final volume of 20 µl containing 11 µl of TaqMan® Fast Advanced Master Mix (ThermoFisher Scientific), primers and probes (Table 1) at a final concentration of 0.25 µM each, and 2 µl of extracted DNA as template. Each reaction also contained DNAs extracted from FDp- and BNp-infected grapevine plants (positive controls), and from two healthy grapevine plants (negative controls). Amplification and detection were done using the 7500 Fast Real-Time PCR System (Applied Biosystems) with optical 96-well plates and adhesive covers. The thermal cycle consisted of a pre-step of 5 min at 95°C for Hot Start Taq DNA polymerase activation, followed by 45 cycles of 60 s denaturation at 95°C and

Table 1. Primer and probe sequences used in real-time PCR for the detection of 16SrV and bois noir phytoplasmas

Set	Name	Sequence 5′→3′	Amplicon size [bp]	Target
I	mapFD-F	TCAAGGCTTCGGBGGTTATA	71	map gene (16SrV-C, D, E phytoplasmas)
	mapFD-R	TTGTTTTAGAAGGTAATCCGTGAACTAC		
	mapFD-FAM	TTGTATTTCAGTGAATGAAG		
	mapBN-F	ATTTGATGAAACACGCTGGATTAA	71	map gene (16SrXII-A phytoplasmas)
	mapBN-R	TCCCTGGAACAATAAAAGTYGCA		
	mapBN-VIC	AAACCCACAAAATGC		
	VITIS-F	AAATTCAGGGAAACCCTGGAA	73	*Vitis* sp. chloroplast tRNAL-F spacer
	VITIS-R	CCCTTGGTTGTTTTCGGAAA		
	VITIS-NED	CTGAGCCAAATCC		
II	FD-F	AAGTCGAACGGAGACCCTTC	103	16S rRNA gene (16SrV phytoplasmas)
	FD-R	TAGCAACCGTTTCCGATTGT		
	FD-Probe (FAM)	AAAAGGTCTTAGTGGCGAACGGGT		
	BN-F	GGTTAAGTCCCGCAACGAG	91	16S rRNA gene (16SrXII-A phytoplasmas)
	BN-R	CCCACCTTCCTCCAATTTATCA		
	BN-Probe (FAM)	AACCCTTGTTGTTAATTGCCATCATTAAG		
	Chaperonin 21-F	GGTCCTTTGGATGAGGATGG	93	chaperonin 21 gene (*Vitis vinifera*)
	Chaperonin 21-R	GAAGTCATTCCCTGCATACTTGG		
	Chaperonin 21-P	GAAACCACTGTCTGTGAGCCCAGGA		

90 s hybridization and elongation at 57°C. The software v2.0 or 2.3 of 7500 fast Real Time PCR system (Applied Biosystems) was used for fluorescence acquisition and evaluation of threshold cycles (Ct). The baseline was automatically set, and the fluorescent threshold was set for each selected target to intercross with the linear part of all amplification curves. In each reaction, amplification goodness was verified by the Ct values of the grapevine endogenous gene target. Samples, in which the grapevine endogenous gene was amplified correctly, were considered infected by 16SrV and/or BN phytoplasma if the amplification threshold cycle (Ct) of the phytoplasma target gene was <37, as previously reported (Mori *et al.* 2015). Infection rate (*IR*) for 16SrV and BN phytoplasmas, obtained with TaqMan® triplex real-time PCR using Set I and Set II, was expressed as a percentage of PCR-positive (infected) samples out of the total analyzed samples. Differences in *IR* (*DIR*) between Set I and II were calculated to establish the most sensitive assay.

Results and Discussion

Description of observed grapevine yellows (GY) symptoms

During field surveys conducted from July to October 2018–2020 in the eastern part of Georgia, 145 out of 8,000 (1.8%) and 147 out of 6,600 (2.2%) grapevine plants, respectively, from mother stock/collection fields and commercial vineyards, were found exhibiting typical or suspicious GY symptoms such as leaf color alterations (yellowing or reddening) and rolling (Figs 1A–D). As reported in a previous study (Quaglino *et al.* 2016), most of the symptomatic grapevine plants of Georgian cultivars showed mild symptoms of GY with no alterations on the berries.

Detection of grapevine yellows (GY) phytoplasmas

Previous studies reported that GY phytoplasmas can be detected in grapevine tissues and insect vector bodies by the utilization of monoclonal and polyclonal antibodies in ELISA tests (Irimia *et al.* 2012; Filippin *et al.* 2019). In the present study, the presence of GY phytoplasmas was not detected in any of the 292 examined grapevine plants from mother stock, collection, and commercial vineyards (data not shown).

On the other hand, TaqMan® triplex real-time PCRs, carried out by protocols published by Pelletier *et al.* (2009) (Set I) and Angelini *et al.* (2007) (Set II), detected GY phytoplasmas (samples with Ct value <37) in 35.3% (103 out of 292) and 13.01% (38 out of 292) of the examined grapevine plants, respectively (Tables 2 and 3). All 38 plants found to be phytoplasma-infected by Set II PCRs were confirmed by Set I PCRs, but 65 plants were found to be infected only by Set I PCRs. GY phytoplasmas were identified in 22.41% (32 out of 145) and in 6.9% (10 out of 145) plants from mother stock and collection vineyards and in 48.3% (71 out of 147) and in 19.0% (28 out of 147) plants from commercial vineyards by Set I and Set II PCRs, respectively (Tables 2 and 3). Detailed results on the molecular detection of 16SrV phytoplasmas and CaPsol in analyzed plants from mother stock and collection vineyards are reported in Tables 2 and 3. Data obtained in the present study showed that PCR protocol by Pelletier *et al.* (2009) was more sensitive than protocol by Angelini *et al.* (2007) in GY phytoplasma detection in the examined plant samples. Even if examined grapevines exhibited typical or suspicious (phytoplasma-like) grapevine yellows symptoms, only 35.3% of such plants were found phytoplasma-infected. This can be due to: (i) the sporadic distribution of phytoplasmas in phloem tissues of infected plants (Constable *et al.* 2003); (ii) the possibility that observed suspicious symptoms are caused by other etiological agents (i.e., viruses) or to abiotic stresses (i.e., chlorosis or other physiological disorders caused by nutritional unbalances) (Belli *et al.* 2010).

Considering the results obtained by Set I PCRs, the average infection rate was 24.1% (ranging from 3.6% in Manavis Mtsvane to 46.9% in Saperavi) and 37.0% (ranging from 11.1% in Crimson sidles to 95.5% in Chardonnay) in Georgian and other varieties, respectively (Tables 1 and 2). As expected from previous studies reporting the wide presence of BN in Georgian vineyards (Quaglino *et al.* 2016), CaPsol was found in most phytoplasma-infected plants (47.6%), with the highest infection rate in Chardonnay, one of the most susceptible varieties exhibiting unambiguously typical GY symptoms (Eveillard *et al.* 2016). Surprisingly, phytoplasmas belonging to taxonomic group 16SrV were detected in 45.6% of the grapevines found phytoplasma-infected. To the best of our knowledge, this is the first report of 16SrV phytoplasmas in Georgia and in the Caucasus region. Considering the neighboring countries, 16SrV phytoplasmas were previously found in association with GY in Turkey and Iran (Ertunc *et al.* 2015; Shahryari *et al.* 2019). It is reasonable to hypothesize that, in Georgia, such 16SrV phytoplasmas could be the ones associated with flavescence dorée. Further molecular typing of detected phytoplasma strains, conducted by sequence analysis of marker genes (*map*, *vmp1*) (Zambon *et al.* 2018; Malembic-Maher *et al.* 2020), is necessary to determine accurately the 16SrV phytoplasma strains detected in grapevines in Georgia in the present study. In Georgia, the grape planting materials are intensively imported from abroad (mainly

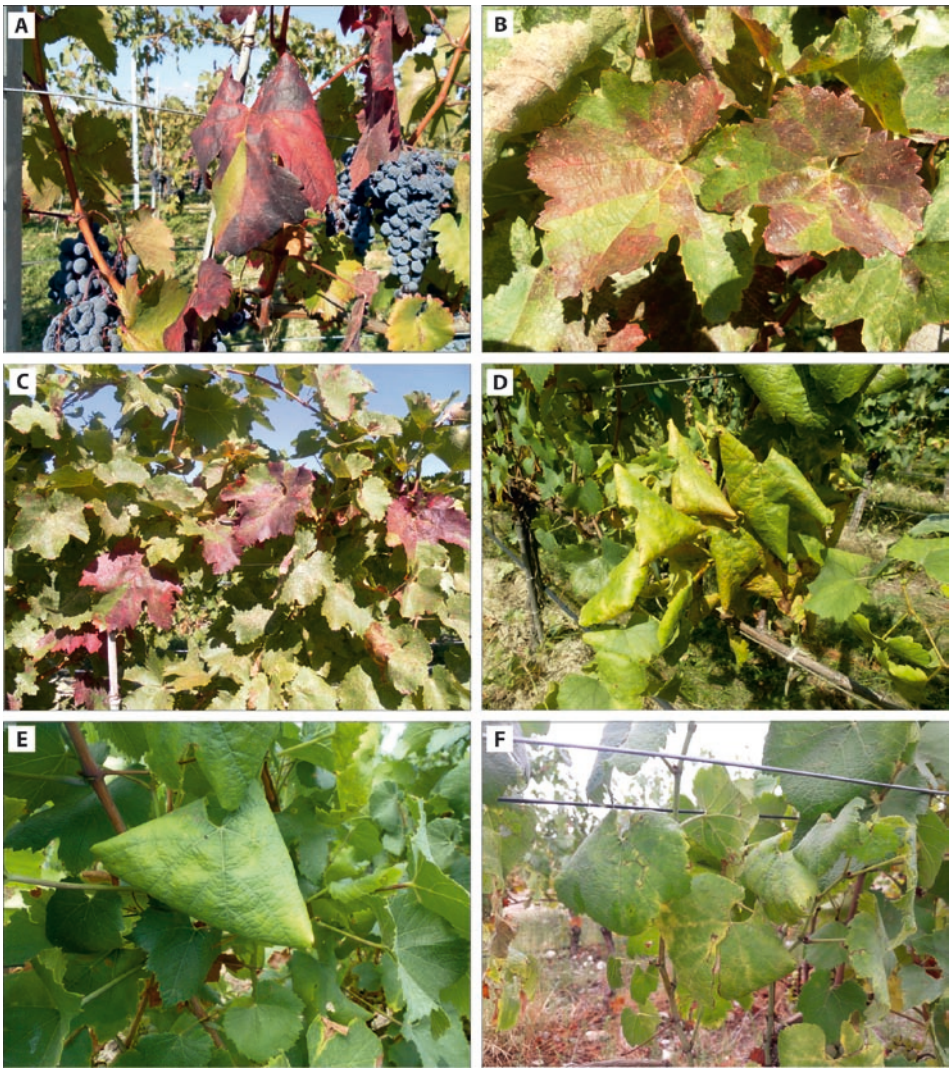

Fig. 1. Grapevine yellows (GY) symptoms observed in red (A, B, C) and white (D, E, F) grapevine varieties in Georgia. A – Saperavi; B – Merlot; C – Cabernet Sauvignon; D – Chardonnay; E – Qistauruli sagvine; F – Khihkvi

Table 2. Detection of 16SrV phytoplasmas and CaPsol in symptomatic grapevines from mother stock and nursery collection vineyards

Cultivar	No. of plants	16SrV		CaPsol		16SrV + CaPsol		No. of infected plants	*IR* [%]
		Infected	Set I–II	Infected	Set I–II	Infected	Set I–II		
Albane Italia	6	1	1–0	0	0–0	0	0–0	1	16.7
Alicante	9	0	0–0	3	3–1	0	0–0	3	33.3
Baga	6	2	2–0	1	1–0	0	0–0	3	50.0
Bastard	6	2	2–1	1	1–1	0	0–0	3	50.0
Black Magic	16	2	2–1	0	0–0	0	0–0	2	12.5
Crimson sidles	54	2	2–2	2	2–1	2	2–1	6	11.1
Grenache	7	1	1–0	1	1–0	0	0–0	2	28.6
Mercy	5	0	0–0	1	1–0	0	0–0	1	20.0
Merlot	18	2	2–0	1	1–0	0	0–0	3	16.7
Qistauruli sagvine	10	1	1–1	0	0–0	0	0–0	1	10.0
Tempranillo	8	2	2–0	4	4–1	1	1–0	7	87.5
Overall	145	15	15–5	14	14–4	3	3–1	32	22.1
	IR [%]	10.3	10.3–3.4	9.7	9.7–2.7	2.1	2.1–0.7		
	Δ*IR* [%]		6.9		6.9		1.4		

IR – infection rate; Δ*IR* – differences in infection rate obtained in Set I and Set II PCRs

Table 3. Detection of 16SrV phytoplasmas and CaPsol in symptomatic grapevines from commercial vineyards

Cultivar	No. of plants	16SrV		CaPsol		16SrV + CaPsol		No. of infected plants	*IR* of [%]
		Infected	Set I–II	Infected	Set I–II	Infected	Set I–II		
Cabernet Sauvignon	18	4	4–2	0	0–0	0	0–0	4	22.2
Chardonnay	44	19	19–13	21	21–9	2	2–2	42	95.5
Khihkvi	25	5	5–2	4	4–3	0	0–0	9	36.0
Manavis Mtsvane	28	0	0–0	1	1–1	0	0–0	1	3.6
Saperavi	32	4	4–3	9	9–3	2	2–1	15	46.9
Overall	147	32	32–20	35	8–5	4	4–3	71	48.3
	IR [%]	21.8	21.8–13.6	23.8	23.8–10.9	2.7	2.7–2.0		
	Δ*IR* [%]		8.2		12.9		0.7		

IR – infection rate; Δ*IR* – differences in infection rate obtained in Set I and Set II PCRs

Italy and France), leading to the risk of new emerging infectious diseases entering the country. Thus, it could be hypothesized that 16SrV phytoplasmas, never identified in Georgia before this study, can be imported into the country by planting material from Europe. It is important to notice that grapevine cultivars Chardonnay, Khihkvi, Saperavi, and Cabernet Sauvignon, found to be infected by 16SrV phytoplasmas, are largely cultivated in Georgia in commercial vineyards (Sargolzaei *et al.* 2021). Among GY, FD phytoplasma is an EPPO quarantine pest which requires permanent field and laboratory monitoring. Testing of rootstock and grafting stock plants for phytoplasma diseases, choosing phytoplasma free plants and removing infected stocks, are useful steps to reduce the spreading of FD and BN and to promote the production of healthy planting material. Thus, the knowledge of typical GY symptoms and the utilization of accurate diagnostic tools are crucial for preventing pathogen spread and producing healthy planting material (Quiroga *et al.* 2020; Nair *et al.* 2021).

In conclusion, based on the results obtained in this study, the presence of BN and 16SrV phytoplasmas was confirmed using two sets of primers and probes in collection and commercial vineyards in the eastern part of Georgia, while the serological method did not reveal phytoplasma infection. Interestingly, the results highlighted the first report of 16SrV phytoplasmas in 11 grapevine varieties in mother stock and collection vineyards and four varieties in commercial vineyards. Monitoring of GY phytoplasma infections should be continued using molecular techniques like triplex real-time PCR.

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