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Development and optimization of a group-specific loop-mediated isothermal amplification (LAMP) assay for the detection of patulin-producing *Penicillium* species

Lisa M. Frisch, Ludwig Niessen*

Technical Microbiology, Technical University of Munich, Gregor-Mendel-Str. 4, 85354 Freising, Germany

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ABSTRACT

The mycotoxin patulin is a toxic fungal secondary metabolite occurring in food worldwide. Methods for rapid, simple and specific detection of patulin-producing fungi in food and feed are therefore urgently needed. In the current study, a loop-mediated isothermal amplification (LAMP) assay based on the isoepoxydon dehydrogenase (*idh*) gene of the patulin biosynthetic pathway was developed and optimized for the group-specific detection of patulin-producing *Penicillium* species. By testing purified DNA of 174 fungal strains representing 31 genera, the assay was demonstrated to be highly specific for the detection of patulin-producing species in *Penicillium*, *Byssochlamys* and *Paecilomyces*. The assay had a detection limit of 2.5 pg of purified genomic DNA of *P. expansum* per reaction. Moreover, the assay was demonstrated to detect patulin-producers when conidia were directly added to the master mix as template without any sample preparation. The applicability of the assay in food analyses was successfully tested on artificially contaminated grapes and apples requiring minimal sample preparation. A screening of grapes from the 2018 harvest from different locations in Germany revealed no presence of patulin-producers. The developed LAMP assay is a promising tool for rapid diagnosis in quality control applications in the food and beverage industry.

1. Introduction

Patulin is a polyketide-derived mycotoxin that presents a potential hazard to food safety and thereby to consumers. Upon its discovery in the early 1940s, it was firstly described as an antibiotic due to its activity against gram-positive and gram-negative bacteria and was hence applied in the treatment of nasal congestion and common cold (Birkinshaw et al., 1943; Chalmers and Clarke, 2004; Hopkins, 1943). However, its toxicity to animals (Broom et al., 1944; Schweitzer, 1946) prevented its further application as pharmacological agent. Patulin shows several different acute and chronic toxicities such as genotoxicity, immunotoxicity, cytotoxicity and mutagenicity (Alves et al., 2000; Bürger et al., 1988; Escoula et al., 1988; Schumacher et al., 2005; Wang et al., 2007). Its main effects in humans are gastrointestinal symptoms, nausea and emesis (Drusch and Ragab, 2003). Because of these harmful effects in humans, many countries worldwide have set limits regarding the patulin content in foods. According to the European Commission (2006), the maximum level of patulin in fruit juices and fermented cider has been set to 50 µg/kg, in solid apple products to 25 µg/kg and in apple juice and solid apple products for infants and young children to 10 µg/kg.

Patulin was found in grapes with *P. expansum* rot and grape products like grape must and juice and also in apple juice (Harwig et al., 1973; Moss, 1998; Scott et al., 1972; Scott et al., 1977). Especially the occurrence on apples is of prime importance worldwide as apples are the most commercialized temperate zone fruit (Da Silva et al., 2007). Moreover, the toxin was also found in pomaceous and stone fruits as well as in their juices and jams (Spadaro et al., 2008). The processing of rotten fruits therefore is the principal cause for patulin contamination in food products (Joshi et al., 2013).

Patulin is produced by several species belonging to *Penicillium, Aspergillus, Byssochlamys* and *Paecilomyces*. Among them, *P. expansum* is considered as the main source of patulin in food affecting public health and causing economic risks (Morales et al., 2008). In the past, it was assumed that patulin can be produced by more than 30 fungal genera (Lai et al., 2000). However, the use of contemporary analytical methods revised the number (Puel et al., 2010) to three *Aspergillus* spp. (*A. clavatus*, *A. giganteus, A. longivesica*) (Varga et al., 2007), 13 *Penicillium* spp. (*P. carneum*, *P. clavigerum*, *P. concentricum*, *P. coprobium*, *P. dipodomyicola*, *P. expansum*, *P. glandicola*, *P. gladioli*, *P. griseofulvum*, *P.*

* Corresponding author.
E-mail address: niessen@wzw.tum.de (L. Niessen).

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marinum, *P. paneum*, *P. sclerotigenum*, *P. vulpinum*) (Frisvad et al., 2004) as well as *Byssochlamys nivea* and some strains of *Paecilomyces saturatus* (Samson et al., 2009).

Since patulin has a negative impact on human health, a rapid and accurate detection of this mycotoxin and its producers in and on food and food products is necessary. The most common method for the detection of mycotoxins combines high-performance liquid chromatography with an ultraviolet light (UV) detector (Moake et al., 2005). However, also traditional methods such as thin-layer chromatography (TLC) or gas chromatography with mass spectrometry (GC–MS) are used (Betina, 1993; Songsermsakul and Razzazi-Fazeli, 2008). Current research is focusing on immunological or aptamer-based assays for faster analysis (Kim et al., 2015; Tomita et al., 2016; Wu et al., 2016). Methods for the detection of patulin are still time-consuming as well as requiring trained personnel and dedicated instrumentation. As a result, the demand of the food industry for rapid detection methods applicable "in-house" without the need for specific equipment is great (Moake et al., 2005).

Detection and identification of patulin-producing molds is currently performed using mainly phenotypic methods including macroscopic analysis and microscopy. The morphological identification is time-consuming and needs profound mycological knowledge and trained staff with considerable expertise to be performed properly. Moreover, it does not invariably provide adequate identification results due to the dependence of growth and sporulation on proper incubation conditions and media to prevent misidentifications (Balajee et al., 2007; Ciardo et al., 2007). Other methods for the identification of molds are the analyses of DNA sequences of reference genes. These methods have become the gold standard for fungal identification during recent years (Summerbell et al., 2005). DNA sequences can also be used for molecular biological diagnosis of patulin-producing fungi. For example, Marek et al. (2003) developed a polymerase chain reaction (PCR) using primers based on the polygalacturonase gene of *P. expansum* and Tannous et al. (2015) developed a real-time PCR assay using primers based on the *patF* gene involved in the patulin biosynthesis. Also Luque et al. (2011) used primers based on a gene involved in the patulin biosynthesis (isoepoxydon dehydrogenase (*idh*)) for PCR-based identification of isolates. A gene probe for the patulin metabolic pathway was based on the *idh* gene as well (Paterson et al., 2010). Although PCR-based methods allow reliable identification and detection of patulin-producers, the preparation of highly purified DNA for the analysis is time-consuming, laborious and expensive. Furthermore, agarose gel electrophoresis is needed for the separation and visualization of the PCR product which represents a big problem when assays are to be applied in on-site investigations in the industry (Niessen and Vogel, 2010).

Loop-mediated isothermal amplification (LAMP) (Notomi, 2000) is a technology for the enzymatic *in vitro* amplification of specific DNA sequences with high specificity, sensitivity and rapidity under isothermal conditions. Its simple handling and the evaluation by in-tube detection of the amplification event together with no need for dedicated equipment make this method advantageous and economically affordable for on-site investigations in the industry. Furthermore, no time-consuming DNA preparation and agarose gel electrophoresis are needed for evaluation of results. Due to its sensitivity, rapidity and specificity, LAMP is a promising tool in a wide range of applications like detection of fungal contaminants and bacterial pathogens (Niessen et al., 2013), surveillance of infectious diseases in developing countries and clinical diagnosis (Fu et al., 2011; Mori and Notomi, 2009). The review of Niessen (2018) provides an overview about LAMP assays for the detection of mycotoxin-producing molds. So far, no LAMP assay has been developed for the group-specific detection of patulin-producing species.

As patulin is a mycotoxin of food safety concern occurring on apples and grapes worldwide, rapid, simple and specific detection of patulin-producing fungi is essential. Therefore, the aim of the current study was the development and optimization of a group-specific LAMP assay for the identification of patulin-producing *Penicillium* species in pure cultures and their detection in contaminated sample materials.

2. Materials and methods

2.1. Fungal cultures and culture conditions

Fungal cultures used in this study are listed in Table 2. Working cultures were grown on malt extract agar plates (MEA, 2% (w/v) malt extract (PanReac AppliChem GmbH, Darmstadt, Germany), 0.2% (w/v) peptone ex soya (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), 1.5% (w/v) agar agar (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), pH 5.6) at ambient temperature (AT, 23 ± 1 °C). For long term preservation, fungal cultures were grown in 50 ml malt extract broth (MEA without agar) with clay granules (Oyaki-Bonsai, Ganzlin, Germany) added without shaking at AT. Granules were transferred to 80% (v/v) sterile glycerol (Gerbu Biotechnik GmbH, Heidelberg, Germany) and stored at −80 °C. For DNA extraction, cultures were grown in 500 µl malt extract broth (MEA without agar) in 1.5 ml reaction vessels for 48 h on a rotary shaker at 170 rpm at AT.

2.2. Preparation of conidial suspensions

Fungal cultures were grown on MEA plates at AT. Conidia were suspended in 5 ml sterile deionized water with 300 µl sterile Tween 20 (Gerbu Biotechnik GmbH, Heidelberg, Germany) and harvested after homogenization of the culture surface with a Drigalski spatula. After centrifugation of the collected suspension (7379 ×*g*, 5 min, AT), the resulting pellet was suspended in 5 ml sterile deionized water. After a second centrifugation step (7379 ×*g*, 5 min, AT), the resulting pellet was stored in 1 ml 50% (v/v) sterile glycerol (Gerbu Biotechnik GmbH, Heidelberg, Germany). A serial dilution was prepared, and total conidial numbers were counted in a hemocytometer (Thoma type, 0.1 mm chamber depth, BRAND GmbH, Wertheim, Germany). Conidial suspensions were stored at 4 °C until used.

2.3. Preparation of template DNA for LAMP

DNA extraction from fungal mycelia was performed according to Cenis (1992) with slight modifications: Grown cultures were washed with 500 µl sterile tap water after centrifugation at 12,470 ×*g* for 5 min at AT. After a second centrifugation step (12,470 ×*g*, 5 min, AT), 300 µl extraction buffer (200 mM Tris-HCl (Gerbu Biotechnik GmbH, Heidelberg, Germany), 250 mM sodium chloride (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), 25 mM EDTA (Gerbu Biotechnik GmbH, Heidelberg, Germany), 0.5% (*w/v*) SDS (SERVA Electrophoresis GmbH, Heidelberg, Germany)) as well as 0.5 g of sterile sea sand (Merck KGaA, Darmstadt, Germany) and 0.1 g of sterile glass beads (Ø 0.5 mm, Scientific Industries, Bohemia, USA) were added before treatment in a FastPrep homogenizer (45 s; 24∗2; 5.5 m/s, MP Biomedicals Germany GmbH, Eschwege, Germany). 150 µl sodium acetate (3 M, pH 5.2 (Carl Roth GmbH & Co. KG, Karlsruhe, Germany)) were added and the tube was placed at −20 °C for 10 min. Following centrifugation (12,470 ×*g*, 5 min, AT), the supernatant was mixed with an equal volume of isopropanol (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) and the tubes were placed at −20 °C for 5 min. The pellet was washed with 500 µl cold 70% ethanol and after two centrifugation steps at 12,470 ×*g* for 5 min at AT, respectively, pellets were dissolved in 50 µl HPLC-grade water for 10 min at 50 °C. The extracted DNA was stored at 4 °C. The DNA concentration was measured in a NanoDrop 1000 spectrophotometer (Peqlab Biotechnologie GmbH, Erlangen, Germany).

The preparation of LAMP templates from contaminated grapes or a grape sample (four grapes per bunch) from the vineyard was done according to Vogt et al. (2017). For contaminated apples, the procedure was slightly modified: 3 ml of sterile tap water containing 1% (*v/v*) Tween 20 (Gerbu Biotechnik GmbH, Heidelberg, Germany) were added

to a third of a contaminated apple in a blender bag (VWR International, Radnor, USA). This mixture was homogenized in a bag mixer (Interscience, Saint-Nom-la-Bretèche, France) and transferred to a 2 ml reaction vessel. The further steps were done according to Vogt et al. (2017). 5 µl of the supernatant were used directly as a template for LAMP reactions or 1:5 diluted to dilute inhibitory substances.

2.4. Artificial contamination of grapes and apples

The washing and artificial contamination of grapes and apples (Gala variety) was performed according to Vogt et al. (2017). Grapes were prick-infected by dipping a sterile tooth pick into a spore suspension (10^8 spores/ml) of *P. expansum* TMW 4.2808 and *P. oxalicum* TMW 4.2539, respectively. The latter fungus is a non-patulin- producer and served as a negative control. Sterile deionized water was used as the non-inoculated control. The same was done with apples after cutting them in three pieces excluding the core. Infected grapes were incubated at AT in sterile 50 ml reaction tubes, each filled with three infected grapes. Infected apples were incubated at AT in sterile blender bags (VWR International, Radnor, USA), each filled with one infected piece.

2.5. DNA amplification

For the neutral red-based LAMP reaction the master mix contained 2.5 µl 10× ammonium sulfate buffer (100 mM ammonium sulfate (Gerbu Biotechnik GmbH, Heidelberg, Germany), 100 mM potassium chloride (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), pH 8.7), 1 µl magnesium chloride (200 mM, Carl Roth GmbH & Co. KG, Karlsruhe, Germany), 3.5 µl dNTPs mix (10 mM each GATC, MP Biomedicals GmbH, Eschwege, Germany), 2.6 µl primer mix (1.6 mM each FIP and BIP, 0.8 mM each LF and LB, 0.2 mM each F3 and B3, see Table 1 for primer sequences), 1 µl *Bst* polymerase (8 U/µl, New England BioLabs GmbH, Frankfurt am Main, Germany), 1 µl neutral red (2.5 mM, SERVA Electrophoresis GmbH, Heidelberg, Germany), 8.4 µl sterile deionized UV treated water and 5 µl of template DNA per 25 µl of reaction volume. To avoid contaminations, two different set of pipettes were used in separate rooms for the preparation of the master mix and for the addition of template DNA. Sterile filter tips were used for all pipetting steps. LAMP reactions were incubated in a thermal cycler (Eppendorf AG, Hamburg, Germany) for 60 min at 65 °C. Subsequently, the reactions were visually checked for a color change from orange to pink indicating a positive reaction. The reactions were photographed for documentation using a hand held mobile phone camera. The activity of the loop primers during DNA amplification was tested in a real-time fluorescence reader (ESEQuant TS, Qiagen, Venlo, The Netherlands). Therefore, the V13-01184 DNA-intercalating dye (Dyomics GmbH, Jena, Germany) was used with 10× MOPS buffer (200 mM MOPS (Gerbu Biotechnik GmbH, Heidelberg, Germany), 100 mM potassium chloride (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), 100 mM ammonium sulfate (Gerbu Biotechnik GmbH, Heidelberg, Germany), pH 8.8) in the LAMP master mix.

To confirm the specificity of the LAMP assay, the nucleotide sequence of the smallest LAMP product of *P. expansum* TMW 4.2808 was analyzed. Therefore, a serial dilution of *P. expansum* TMW 4.2808 genomic DNA was amplified by LAMP and the products were loaded on an agarose gel. The smallest amplified DNA fragments visible in all positive reactions were excised from the gel and sequenced using the primers F2-IDH and B2-IDH (see Table 1).

Table 1
List of primers used in the current study. Wobble bases are defined as Y (C/T), N (A/C/G/T), R (G/A) and V (G/A/C).

Primer name	Sequence 5′-3′
F3-IDH-ID5	AGTTTYGCGATCGATGTCAT
B3-IDH-ID5	CTTNGGCCCYAAGAAGTGG
FIP-IDH-ID5	TGGATGCCTGGGGRGACTTT-GGGYTTCGTYGAGCTGGT
BIP-IDH-ID5	CGGGAATTCTACCGGTCCCCT-CAATTCCTGVACATGCTGC
LF-IDH-ID5	AGTAGGGAGTAGCCGCCTT
LB2-IDH	GCAGCCTACGGGCCCTGC
F2-IDH	GGGYTTCGTYGAGCTGGT
B2-IDH	CAATTCCTGVACATGCTGC

2.6. Agarose gel electrophoresis

A 1.3% (w/v) agarose (Biozym Scientific GmbH, Hessisch Oldendorf, Germany) gel prepared with 1× TAE buffer (2 M Tris-HCl (Gerbu Biotechnik GmbH, Heidelberg, Germany), 0.1 M EDTA (Gerbu Biotechnik GmbH, Heidelberg, Germany), 1 M acetic acid (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), pH 8.2) was used at 110 V for 60 min at AT. 5 µl of the DNA sample mixed with 1 µl 6× DNA loading dye (Thermo Fisher Scientific Inc., Waltham, USA) and 6 µl of size marker (Gene Ruler 100 bp Plus DNA Ladder, Thermo Fisher Scientific Inc., Waltham, USA) were loaded in the gel pockets. For staining, gels were immersed in an aqueous solution of dimidium bromide (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) for 20 min and subsequently washed in deionized water for 15 min. Gels were inspected under UV light (365 nm) and photographed using a video documentation system (Intas Science Imaging Instruments GmbH, Göttingen, Germany).

3. Results

3.1. Design of LAMP primers and confirmation of the LAMP product

LAMP primers were designed using the PrimerExplorer V.5 software tool (http://primerexplorer.jp/e) provided by Eiken Chemical Co., Ltd. (Tokyo, Japan). The primer set IDH-ID5 was based on an alignment of isoepoxydon dehydrogenase (*idh*) gene sequences of the patulin biosynthetic pathway of *Penicillium expansum* (NRRL 6069), *Penicillium gladioli* (NRRL 938), *Penicillium sclerotigenum* (NRRL 22813), *Penicillium vulpinum* (NRRL 2031), *Penicillium carneum* (NRRL 25170), *Penicillium concentricum* (NRRL 2034), *Penicillium paneum* (NRRL 25162), *Penicillium dipodomyicola* (NRRL 35583), *Penicillium clavigerum* (NRRL 1004), *Penicillium coprobium* (NRRL 13626), *Penicillium glandicola* (NRRL 985) and *Penicillium griseofulvum* (NRRL 5256). The position and orientation of the used primers is shown in Fig. 1. In order to detect a broad spectrum of potential patulin-producers, wobble positions were introduced in the primers where nucleotides varied within a given position. The specificity of the designed primers was tested and confirmed *in silico* by the nucleotide BLAST search tool on the NCBI database (Altschul et al., 1990).

To confirm DNA amplification in positive LAMP reactions, the LAMP products of a tenfold serial dilution of purified DNA of *P. expansum* TMW 4.2808 were separated on an agarose gel. Fig. 2 shows the typical ladder-like banding pattern in the agarose gel caused by positive reactions in the LAMP assay. In order to confirm the specificity of the LAMP assay and the identity of amplification products, the smallest DNA fragment occurring in positive reactions was excised and sequenced using primers F2-IDH and B3-IDH (see Table 1). The sequencing results showed 96% identity of the obtained sequence with the *idh* gene of different *P. expansum* strains (NRRL 35695, NRRL 6069, NRRL 35231, NRRL 2304, NRRL 35259, NRRL 32293, NRRL 32289) according to the NCBI database.

3.2. Optimization and characterization of the LAMP assay

To determine the optimum incubation temperature, LAMP reactions were run in a thermal cycler that provided a temperature gradient for isothermal incubation using neutral red as indicator dye. Positive reactions were identified by a color change from orange to pink. Using 5 ng per reaction of purified DNA from *P. expansum* TMW 4.2808, LAMP reactions were incubated at individual temperatures ranging

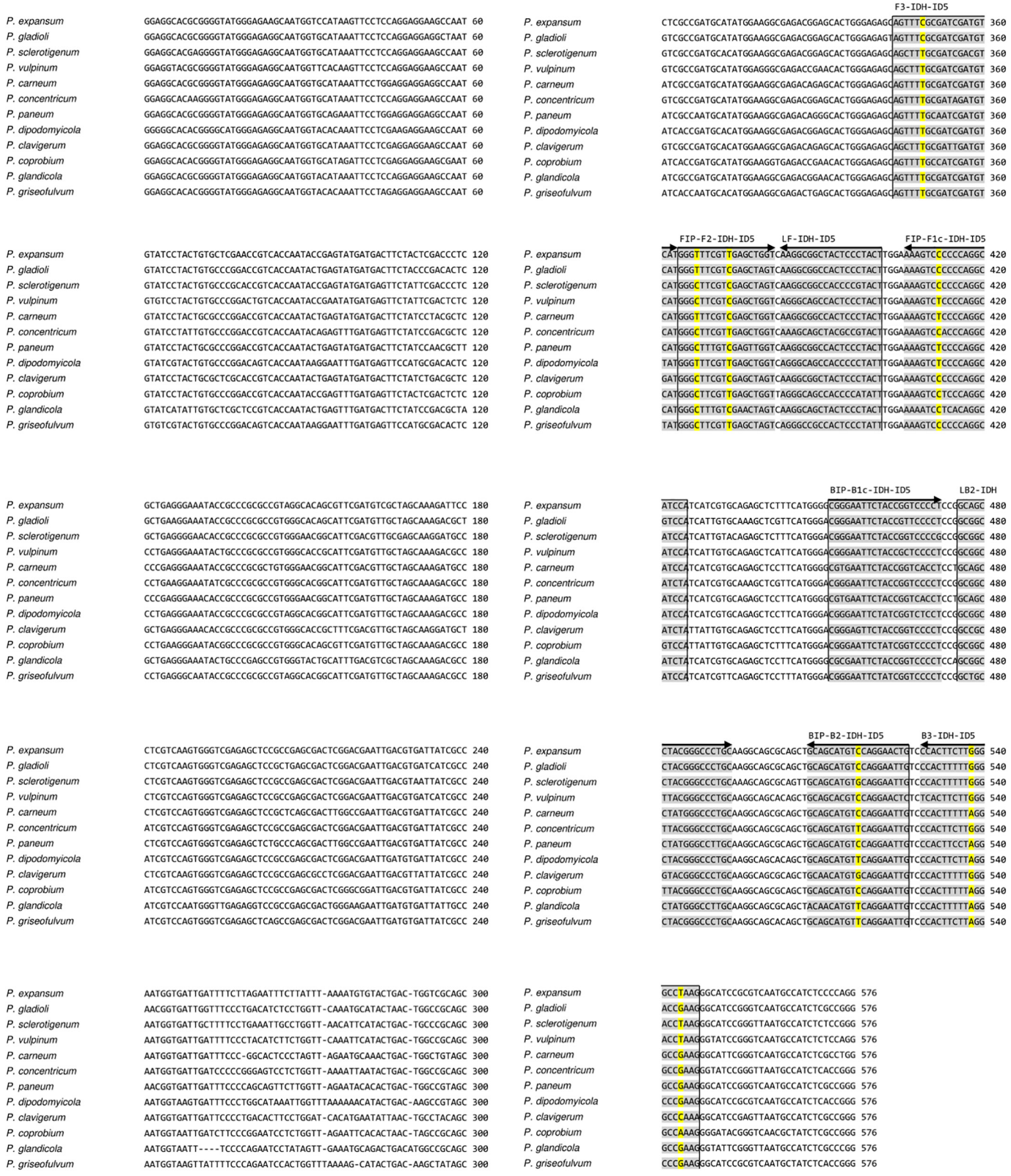

Fig. 1. Alignment of *idh* gene sequences of patulin-producing *Penicillium* species. The position and orientation of designed LAMP primers are highlighted in grey and with arrows. Wobble positions are highlighted in yellow. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

from 59 °C to 71.5 °C in twelve increments. After 60 min, a positive signal was found in all reactions incubated at temperatures between 59 °C and 67.2 °C (see Fig. 3). Higher temperatures inhibited the LAMP assay completely. Incubation at temperatures between 63.9 °C and 65.6 °C led to a positive result already after 30 min. According to these findings, 65 °C was chosen as isothermal incubation temperature for all further experiments.

The sensitivity of the developed assay was assessed by testing

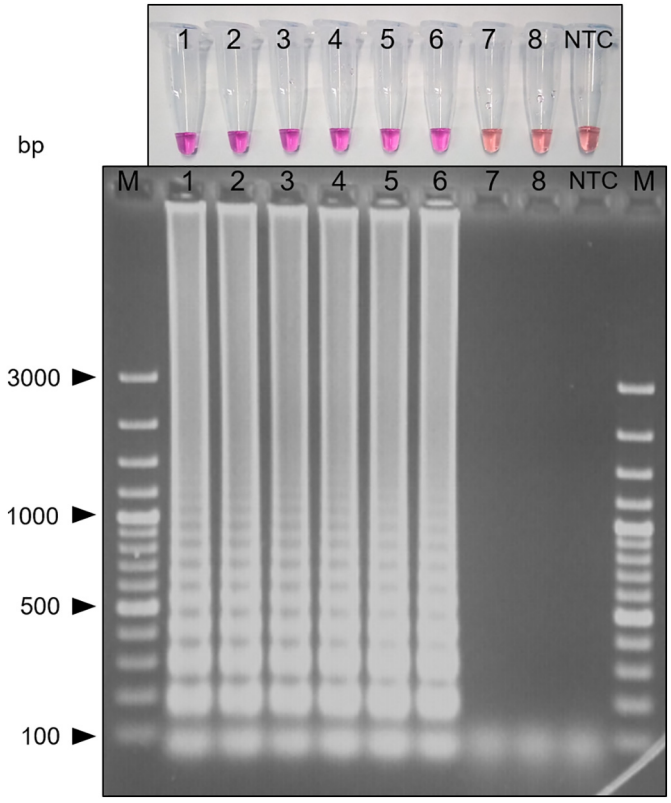

Fig. 2. Analysis of LAMP amplification products by electrophoresis. LAMP assay with a tenfold serial dilution of purified DNA of *P. expansum* TMW 4.2808 ranging from 250 ng/reaction (rxn) to 25 fg/rxn. Pink = positive reaction, orange = negative reaction. 1 = 250 ng/rxn, 2 = 25 ng/rxn, 3 = 2.5 ng/rxn, 4 = 250 pg/rxn, 5 = 25 pg/rxn, 6 = 2.5 pg/rxn, 7 = 250 fg/rxn, 8 = 25 fg/rxn, NTC = no template control with water instead of DNA, M = size marker (Gene Ruler 100 bp Plus DNA Ladder, Thermo Fisher Scientific Inc., Waltham, USA), bp = base pairs. LAMP products were applied in the same order on an agarose gel. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

tenfold serial dilutions of purified DNA of *P. expansum* TMW 4.2808 ranging from 250 ng per reaction to 25 fg per reaction. The results are shown in Fig. 4. A positive result occurred in reactions with 250 ng/rxn to 2.5 pg/rxn. No positive result was visible in the reactions with 250 fg/rxn to 25 fg/rxn. The detection minimum was therefore 2.5 pg/rxn. According to the NCBI database, the genome of *P. expansum* (MD-8) has a size of 32.35 Mbp. Since *idh* is a single copy gene, the minimum detected amount of DNA is corresponding to 71.6 genome copies. Furthermore, serially diluted spore suspensions of *P. expansum* TMW 4.2808 ranging from 1×10^6 to 1×10^1 spores per reaction were tested in the LAMP assay with regard to assay sensitivity. The results are shown in Fig. 5. Positive reactions were visible with 1×10^6 spores per reaction to 1×10^3 spores per reaction. No positive results occurred in reactions that had 1×10^2 or less spores per reaction.

The specificity of the assay was determined by testing purified DNA (100 ng/reaction) of 174 fungal strains representing 31 genera, as well as by adding spores of 62 fungal strains directly from the agar plate to the LAMP assay, including typical patulin-producers. The results are listed in Table 2. All available and tested patulin-producers (typed in bold letters), except for strains of *A. clavatus,* provoked positive results. Among the non-patulin-producers, *P. roqueforti* TMW 4.1599 and *P. coprophilum* TMW 4.2815 showed a positive reaction.

The activity of loop primers during DNA amplification was tested in a real-time fluorescence reader. LAMP reactions with purified DNA of *P. expansum* TMW 4.2808 (250 ng per reaction) and different sets of IDH-primers containing both loop primers, no loop primers and either forward or backward loop primer were tested by incubation at 65 °C for 90 min using the intercalating dye V13-01184 for signal generation. Results revealed that with both forward and backward loop primers added, the on-set of the amplification signal started after 13 min. Without the addition of any loop primers, earliest signals occurred after 39 min. The on-set with only the backward loop primer started after 16 min and with only the forward loop primer after 18 min.

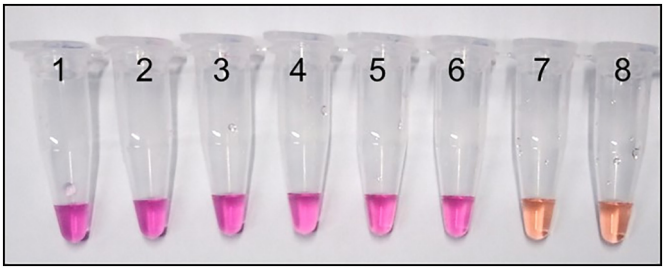

Fig. 4. LAMP assay with a tenfold serial dilution of purified DNA of *P. expansum* TMW 4.2808 ranging from 250 ng/reaction (rxn) to 25 fg/rxn. Pink = positive reaction, orange = negative reaction. 1 = 250 ng/rxn, 2 = 25 ng/rxn, 3 = 2.5 ng/rxn, 4 = 250 pg/rxn, 5 = 25 pg/rxn, 6 = 2.5 pg/rxn, 7 = 250 fg/rxn, 8 = 25 fg/rxn. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

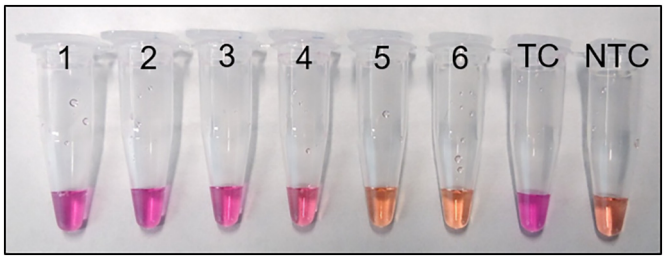

Fig. 5. LAMP assay with a tenfold serial dilution of a spore suspension of *P. expansum* TMW 4.2808 ranging from 1×10^6 to 1×10^1 spores per reaction (rxn). Pink = positive reaction, orange = negative reaction. 1 = 1×10^6 spores/rxn, 2 = 1×10^5 spores/rxn, 3 = 1×10^4 spores/rxn, 4 = 1×10^3 spores/rxn, 5 = 1×10^2 spores/rxn, 6 = 1×10^1 spores/rxn, TC = template control with purified DNA of *P. expansum* TMW 4.2808 (50 ng/µl), NTC = no template control with water instead of DNA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

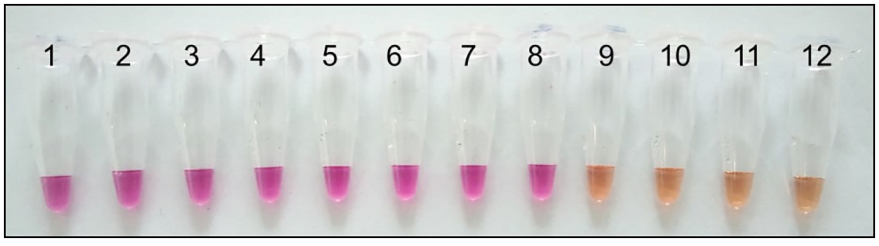

Fig. 3. LAMP assay with a temperature gradient from 59 °C to 71.5 °C. Pink = positive reaction, orange = negative reaction. LAMP with purified DNA of *P. expansum* TMW 4.2808 incubated at 1 = 59 °C, 2 = 59.2 °C, 3 = 59.9 °C, 4 = 60.9 °C, 5 = 62.3 °C, 6 = 63.9 °C, 7 = 65.6 °C, 8 = 67.2 °C, 9 = 68.7 °C, 10 = 70 °C, 11 = 71 °C; 12 = 71.5 °C. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Fungal strains used in the current study and their reactions in the LAMP assay by testing with purified DNA (100 ng/reaction) of 174 fungal strains and spores of 62 fungal strains directly taken from agar plates. + = positive reaction, − = negative reaction, n.t. = not tested. Patulin-producers (according to literature) are typed in bold letters.

Species	Strain	Clone	LAMP reaction	
			DNA	Spores
Alternaria alternata	TMW 4.0438[a]	TMW 4.0438	−	n.t.
Alternaria mali	CBS 106.24[c]	TMW 4.1406	−	n.t.
Alternaria spp.	TMW 4.1428	TMW 4.1428	−	n.t.
Aspergillus aculeatus	TMW 4.1776	TMW 4.1776	−	n.t.
Aspergillus alliaceus	DSM 813[e]	TMW 4.1077	−	n.t.
Aspergillus arachidicola	IBT 27128[f]	TMW 4.2204	−	n.t.
Aspergillus auricomus	CBS 467.65	TMW 4.1631	−	n.t.
Aspergillus awamori	CBS 101.704	TMW 4.1066	−	n.t.
Aspergillus bombycis	IBT 23536	TMW 4.2210	−	n.t.
Aspergillus bridgeri	CBS 350.81	TMW 4.1632	−	n.t.
Aspergillus caelatus	IBT 29700	TMW 4.2209	−	n.t.
Aspergillus carbonarius	TMW 4.1512	TMW 4.1512	−	n.t.
Aspergillus clavatus	CBS 513.65	TMW 4.1086	−	−
Aspergillus clavatus	IBT 12362	TMW 4.1976	−	−
Aspergillus clavatus	IBT 12778	TMW 4.1977	−	−
Aspergillus clavatus	IBT 18790	TMW 4.1978	−	−
Aspergillus clavatus	IBT 21704	TMW 4.1979	−	−
Aspergillus clavatus	IBT 21863	TMW 4.1980	−	−
Aspergillus elegans	CBS 310.80	TMW 4.1633	−	n.t.
Aspergillus ellipticus	CBS 707.79	TMW 4.1629	−	n.t.
Aspergillus flavus	TMW 4.1859	TMW 4.1859	−	n.t.
Aspergillus foetidus	CBS 114.49	TMW 4.1628	−	n.t.
Aspergillus fumigatus	CBS 113.55	TMW 4.0623	−	n.t.
Aspergillus helicothrix	CBS 677.79	TMW 4.1630	−	n.t.
Aspergillus heteromorphus	CBS 117.55	TMW 4.1626	−	n.t.
Aspergillus insulicola	CBS 382.75	TMW 4.1634	−	n.t.
Aspergillus japonicus	CBS 114.51	TMW 4.1627	−	n.t.
Aspergillus minisclerotigenes	IBT 27177	TMW 4.2205	−	n.t.
Aspergillus niger	CBS 101.698	TMW 4.1068	−	n.t.
Aspergillus nomius	CBS 260.88	TMW 4.1960	−	n.t.
Aspergillus ochraceoroseus	CBS 101.887	TMW 4.1772	−	n.t.
Aspergillus ochraceus	CBS 263.67	TMW 4.0706	−	n.t.
Aspergillus oryzae	IBT 28103	TMW 4.2208	−	n.t.
Aspergillus parasiticus	CBS 126.62	TMW 4.1768	−	n.t.
Aspergillus parvisclerotigenes	IBT 3850	TMW 4.2205	−	n.t.
Aspergillus petrakii	CBS 105.57	TMW 4.1087	−	n.t.
Aspergillus pseudotararii	IBT 21092	TMW 4.2212	−	n.t.
Aspergillus rambellii	IBT 14580	TMW 4.2211	−	n.t.
Aspergillus sclerotiorum	CBS 549.65	TMW 4.1089	−	n.t.
Aspergillus sojae	IBT 21643	TMW 4.2207	−	n.t.
Aspergillus sulfureus	CBS 550.65	TMW 4.1067	−	n.t.
Aspergillus tamarii	CBS 591.68	TMW 4.1771	−	n.t.
Aspergillus terreus	CBS 377.64	TMW 4.1060	−	n.t.
Aspergillus toxicarius	CBS 822.72	TMW 4.1766	−	n.t.
Aspergillus tubingensis	ITEM 4496[g]	TMW 4.2008	−	n.t.
Aspergillus usamii var. *shiro-usamii*	CBS 101.700	TMW 4.1072	−	n.t.
Aureobasidium pullulans	TMW 4.2253	TMW 4.2253	−	n.t.
Beltraniella portoricensis	CBS 856.70	TMW 4.0402	−	n.t.
Bipolaris sorokiniana	CBS 311.64	TMW 4.0509	−	n.t.
Byssochlamys nivea	TMW 4.1565	TMW 4.1565	+	+
Byssochlamys nivea	CBS 100.11	TMW 4.1594	+	+
Cladobotryum dendroides	NRRL 2903[j]	TMW 4.0467	−	n.t.
Cladosporium macrocarpum	TMW 4.2371	TMW 4.2371	−	n.t.
Cladosporium sphaerospermum	TMW 4.2370	TMW 4.2370	−	n.t.
Colletotrichum acutatum	CBS 295.67	TMW 4.0652	−	n.t.
Colletotrichum fragariae	CBS 142.31	TMW 4.0651	−	n.t.
Colletotrichum gloeosporioides	CBS 285.50	TMW 4.0650	−	n.t.
Cryptomela acutispora	CBS 157.33	TMW 4.1620	−	n.t.
Drechslera teres	CBS 378.59	TMW 4.0558	−	n.t.
Drechslera tricici-repentis	CBS 265.80	TMW 4.0559	−	n.t.
Emericella astellata	IBT 21903	TMW 4.2202	−	n.t.
Emericella olivicola	IBT 26499	TMW 4.2201	−	n.t.
Emericella venezuelensis	IBT 20956	TMW 4.2203	−	n.t.
Epicoccum nigrum	TMW 4.1407	TMW 4.1407	−	n.t.
Fusarium acuminatum	CBS 485.94	TMW 4.0701	−	n.t.
Fusarium avenaceum	DSM 62161	TMW 4.0140	−	n.t.
Fusarium beomiforme	BBA 69406[a]	TMW 4.0513	−	n.t.
Fusarium cerealis	CBS 589.93	TMW 4.0406	−	n.t.
Fusarium chlamydosporum	CBS 145.25	TMW 4.0404	−	n.t.
Fusarium compactum	CBS 466.92	TMW 4.0433	−	n.t.

(*continued on next page*)

Table 2 (*continued*)

Species	Strain	Clone	LAMP reaction	
			DNA	Spores
Fusarium culmorum	DSM 62191	TMW 4.0149	−	n.t.
Fusarium dimerum	CBS 175.31	TMW 4.0626	−	n.t.
Fusarium dlaminii	MRC 3024[h]	TMW 4.0571	−	n.t.
Fusarium equiseti	CBS 406.86	TMW 4.0477	−	−
Fusarium eumartii	DSM 62809	TMW 4.0303	−	n.t.
Fusarium heterosporum	DSM 62231	TMW 4.0224	−	n.t.
Fusarium longipes	CBS 739.79	TMW 4.0350	−	n.t.
Fusarium melanochlorum	CBS 202.65	TMW 4.0625	−	n.t.
Fusarium napiforme	BBA 67629	TMW 4.0510	−	n.t.
Fusarium oxysporum	DSM 62292	TMW 4.0163	−	n.t.
Fusarium proliferatum	DSM 62261	TMW 4.0236	−	n.t.
Fusarium scirpi	CBS 448.84	TMW 4.0410	−	n.t.
Fusarium solani	DSM 62416	TMW 4.0255	−	n.t.
Fusarium subglutinans	BBA 63621	TMW 4.0947	−	n.t.
Fusarium sublunatum var. *sublunatum*	CBS 189.34	TMW 4.0417	−	n.t.
Fusarium torulosum	BBA 64465	TMW 4.0437	−	n.t.
Geomyces auratus	BBA 66636	TMW 4.0904	−	n.t.
Geomyces auratus	BBA 66873	TMW 4.0905	−	n.t.
Geomyces auratus	BBA 66886	TMW 4.0906	−	n.t.
Geomyces pannorum	BBA 66108	TMW 4.0902	−	n.t.
Geomyces pannorum	TMW 4.2074	TMW 4.2074	−	n.t.
Geomyces pannorum	TMW 4.2340	TMW 4.2340	−	n.t.
Geotrichum candidum	TMW 4.0508	TMW 4.0508	−	n.t.
Gliocephalotrichum spec. nov.	NRRL 2993	TMW 4.0468	−	n.t.
Hypomyces rosellus	CBS 521.81	TMW 4.0400	−	n.t.
Memnoniella echinata	CBS 627.61	TMW 4.0711	−	n.t.
Microdochium majus	TMW 4.0496	TMW 4.0496	−	n.t.
Microdochium nivale	TMW 4.0495	TMW 4.0495	−	n.t.
Monascus ruber	TMW 4.1426	TMW 4.1426	−	n.t.
Mucor hiemalis	TMW 4.2319	TMW 4.2319	−	n.t.
Mucor mucedo	DSM 809	TMW 4.0441	−	n.t.
Myrothecium roridum	CBS 331.51	TMW 4.0668	−	n.t.
Paecilomyces saturatus	TMW 4.2614	TMW 4.2614	+	+
Penicillium aurantiogriseum	CBS 225.90	TMW 4.1603	−	n.t.
Penicillium brevicompactum	TMW 4.2279	TMW 4.2279	−	n.t.
Penicillium camembertii	DSM 1233	TMW 4.0442	−	n.t.
Penicillium chrysogenum	CBS 573.68	TMW 4.1958	−	n.t.
Penicillium clavigerum	TMW 4.1973	TMW 4.1973	+	+
Penicillium clavigerum	TMW 4.1974	TMW 4.1974	+	+
Penicillium clavigerum	TMW 4.1975	TMW 4.1975	+	+
Penicillium commune	CBS 311.48	TMW 4.1088	−	n.t.
Penicillium commune	TMW 4.2270	TMW 4.2270	−	n.t.
Penicillium coprophilum	SP 817[m]	TMW 4.2815	+	+
Penicillium corylophilum	CBS 321.48	TMW 4.1598	−	n.t.
Penicillium crustosum	CBS 499.73	TMW 4.1080	−	n.t.
Penicillium digitatum	DSM 62840	TMW 4.1083	−	n.t.
Penicillium expansum	DSM 62841	TMW 4.0466	+	+
Penicillium expansum	TMW 4.1363	TMW 4.1363	+	+
Penicillium expansum	TMW 4.2495	TMW 4.2495	+	+
Penicillium expansum	TMW 4.2496	TMW 4.2496	+	+
Penicillium expansum	ITEM 6801	TMW 4.2577	+	+
Penicillium expansum	ITEM 7015	TMW 4.2578	+	+
Penicillium expansum	ITEM 7545	TMW 4.2579	+	+
Penicillium expansum	ITEM 9590	TMW 4.2580	+	+
Penicillium expansum	TMW 4.2778	TMW 4.2778	+	+
Penicillium expansum	TMW 4.2779	TMW 4.2779	+	+
Penicillium expansum	TMW 4.2780	TMW 4.2780	+	+
Penicillium expansum	TMW 4.2781	TMW 4.2781	+	+
Penicillium expansum	[i]MUM 17.87	TMW 4.2802	+	+
Penicillium expansum	MUM 17.88	TMW 4.2803	+	+
Penicillium expansum	MUM 17.86	TMW 4.2804	+	+
Penicillium expansum	TMW 4.2805	TMW 4.2805	+	+
Penicillium expansum	TMW 4.2806	TMW 4.2806	+	+
Penicillium expansum	TMW 4.2807	TMW 4.2807	+	+
Penicillium expansum	TMW 4.2808	TMW 4.2808	+	+
Penicillium expansum	TMW 4.2820	TMW 4.2820	n.t.	+
Penicillium expansum	TMW 4.2821	TMW 4.2821	n.t.	+
Penicillium expansum	TMW 4.2822	TMW 4.2822	n.t.	+
Penicillium expansum	TMW 4.2823	TMW 4.2823	n.t.	+
Penicillium expansum	TMW 4.2824	TMW 4.2824	n.t.	+
Penicillium expansum	TMW 4.2825	TMW 4.2825	n.t.	+
Penicillium expansum	TMW 4.2826	TMW 4.2826	n.t.	+
Penicillium expansum	TMW 4.2827	TMW 4.2827	n.t.	+

(*continued on next page*)

Table 2 (*continued*)

Species	Strain	Clone	LAMP reaction	
			DNA	Spores
Penicillium expansum	TMW 4.2828	TMW 4.2828	n.t.	+
Penicillium expansum	TMW 4.2829	TMW 4.2829	n.t.	+
Penicillium expansum	TMW 4.2830	TMW 4.2830	n.t.	+
Penicillium expansum	TMW 4.2831	TMW 4.2831	n.t.	+
Penicillium expansum	TMW 4.2832	TMW 4.2832	n.t.	+
Penicillium expansum	TMW 4.2833	TMW 4.2833	n.t.	+
Penicillium expansum	TMW 4.2834	TMW 4.2834	n.t.	+
Penicillium expansum	TMW 4.2835	TMW 4.2835	n.t.	+
Penicillium expansum	TMW 4.2836	TMW 4.2836	n.t.	+
Penicillium expansum	TMW 4.2837	TMW 4.2837	n.t.	+
Penicillium glabrum	TMW 4.2027	TMW 4.2027	−	n.t.
Penicillium glandicola	TMW 4.2499	TMW 4.2499	+	+
Penicillium glandicola	TMW 4.2500	TMW 4.2500	+	+
Penicillium griseofulvum	TMW 4.1543	TMW 4.1543	+	+
Penicillium griseofulvum	MUM 14.34	TMW 4.2532	+	+
Penicillium italicum	DSM 62846	TMW 4.1084	−	n.t.
Penicillium janthinellum	TMW 4.2318	TMW 4.2318	−	−
Penicillium jensenii	TMW 4.2316	TMW 4.2316	−	n.t.
Penicillium nalgiovense	TMW 4.1371	TMW 4.1371	−	n.t.
Penicillium nordicum	BFE 487[b]	TMW 4.2213	−	n.t.
Penicillium nordicum	TMW 4.2271	TMW 4.2271	−	n.t.
Penicillium olsonii	TMW 4.1362	TMW 4.1362	−	−
Penicillium oxalicum	MUM 14.41	TMW 4.2539	−	−
Penicillium oxalicum	MUM 17.82	TMW 4.2799	−	n.t.
Penicillium oxalicum	MUM 17.81	TMW 4.2800	−	n.t.
Penicillium paneum	MUM 14.47	TMW 4.2542	+	+
Penicillium purpurescens	CBS 223.28	TMW 4.1082	−	n.t.
Penicillium purpurogenum	CBS 286.36	TMW 4.1079	−	n.t.
Penicillium roqueforti	CBS 221.30	TMW 4.1599	+	+
Penicillium roseopurpureum	TMW 4.1770	TMW 4.1770	−	n.t.
Penicillium rugulosum	TMW 4.1902	TMW 4.1902	−	n.t.
Penicillium stoloniferum	TMW 4.2280	TMW 4.2280	−	n.t.
Penicillium variabile	CBS 385.48	TMW 4.1081	−	n.t.
Penicillium verrucosum	CBS 603.74	TMW 4.1073	−	n.t.
Penicillium vulpinum	TMW 4.1605	TMW 4.1605	+	+
Penicillium vulpinum	TMW 4.2399	TMW 4.2399	+	+
Penicillium waksmanii	TMW 4.2317	TMW 4.2317	−	n.t.
Pseudogymnoascus destructans	OT-24-2010[k]	only DNA	−	n.t.
Pseudogymnoascus destructans	OT-26-2010	TMW 4.2513	−	n.t.
Pseudogymnoascus roseus	CCF 3426[d]	TMW 4.2421	−	n.t.
Scopulariopsis acremonioides	TMW 4.2366	TMW 4.2366	−	n.t.
Scopulariopsis brevicaulis	TMW 4.2368	TMW 4.2368	−	n.t.
Stachybotrys chartarum	Sp 2682[l]	TMW 4.0523	−	n.t.
Trichoderma harzianum	TMW 4.1502	TMW 4.1502	−	n.t.
Trichoderma virens	CBS 344.47	TMW 4.0710	−	n.t.
Trichothecium roseum	CBS 567.50	TMW 4.0691	−	n.t.
Zygosaccharomyces bailii	DSM 70834	TMW 3.058	−	n.t.
Zygosaccharomyces bisporus	TMW 3.062	TMW 3.062	−	n.t.
Zygosaccharomyces rouxii	DSM 2531	TMW 3.057	−	n.t.

[a] BBA = Julius Kühn-Institut, Bundesforschungsinstitut für Kulturpflanzen, Berlin, GER.
[b] BFE = Max Rubner-Institut, Bundesforschungsinstitut für Ernährung und Lebensmittel, Karlsruhe, GER.
[c] CBS = Westerdijk Fungal Biodiversity Institute, Utrecht, NL.
[d] CCF = Culture Collection of Fungi, Charles University Prague, CZE.
[e] DSM = Deutsche Sammlung von Mikroorganismen und Zellkulturen, Darmstadt, GER.
[f] IBT = Culture collection of Center for Microbial Biotechnology (CMB), Department of Systems Biology, Technical University of Denmark, Lyngby, DK.
[g] ITEM = Istituto Tossine e Micotossine da Parassiti Vegetali, CNR, Bari, IT.
[h] MRC = South African Medical Research Council, Tygerberg, SA.
[i] MUM = Micoteca da Universidade Minho, University of Minho, Braga, PT.
[j] NRRL = Northern Regional Research Laboratory, Peoria (Illinois), USA.
[k] OT = Leibniz-Institut für Zoo- und Wildtierforschung, Berlin, GER.
[l] Sp = Max Rubner-Institut, Bundesforschungsinstitut für Ernährung und Lebensmittel, Kulmbach, GER.
[m] SP = Lehrstuhl für Lebensmittelsicherheit, Ludwig-Maximilians-University, Munich, GER.
[n] TMW = Lehrstuhl für Technische Mikrobiologie Weihenstephan, Technical University of Munich, Freising, GER.

3.3. Application of the LAMP assay to artificially contaminated grapes and apples

Over a period of ten days, the LAMP assay was tested on artificially contaminated grapes and apples. Grapes and apples were treated and analyzed as described in Sections 2.3 and 2.4. The results are shown in Table 3. Positive signals for *P. expansum* were detected at day two and following days in grapes and at day four and following days in apples. No signals were obtained in LAMP reactions run with fruits infected with *P. oxalicum* as a non-patulin-producing fungus as well as with sterile deionized water ($_dH_2O$) as negative control.

Table 3
Results of the LAMP assay with artificially contaminated grapes and apples.

Results of the LAMP assay with artificially contaminated grapes and apples over a period of ten days. The fruits were prick-infected with *P. expansum* TMW 4.2808, *P. oxalicum* TMW 4.2539 (10^8 spores/ml) and sterile deionized water ($_dH_2O$) was used as negative control. + = positive reaction, − = negative reaction.

	Grapes			Apples		
Day	$_dH_2O$	*P. oxalicum* TMW 4.2539	*P. expansum* TMW 4.2808	$_dH_2O$	*P. oxalicum* TMW 4.2539	*P. expansum* TMW 4.2808
0	−	−	−	−	−	−
1	−	−	−	−	−	−
2	−	−	+	−	−	−
3	−	−	+	−	−	−
4	−	−	+	−	−	+
5	−	−	+	−	−	+
6	−	−	+	−	−	+
7	−	−	+	−	−	+
8	−	−	+	−	−	+
9	−	−	+	−	−	+
10	−	−	+	−	−	+

3.4. Detection of patulin-producing Penicillium species on grape samples of various origin

28 grape samples from the 2018 harvest (each containing two bunches of grapes) of different varieties and locations in Germany were tested with the developed LAMP assay using the same sample preparation protocol as for artificially contaminated samples. All samples, 20 Riesling, two Traminer, two Chardonnay white and two Chardonnay rose from Geisenheim, one Pinot Blanc from Selzen and one Johanniter from Baden, provoked no positive LAMP results indicating no presence of patulin-producing *Penicillium* spp. on them.

4. Discussion

Patulin is a mycotoxin of food safety concern occurring in food worldwide. Since a rapid, simple and specific detection of patulin-producing fungi is essential for quality control in the food and feed industry, a group-specific LAMP assay was developed and optimized for the identification and detection of patulin-producing *Penicillium* species in pure cultures and in contaminated sample materials.

The LAMP primers used in this study were based on the isoepoxydon dehydrogenase (*idh*) gene that is involved in the patulin biosynthetic pathway. Dombrink-Kurtzman (2007) showed a high sequence similarity in this gene between twelve different *Penicillium* spp. The findings were confirmed by the alignment performed in the current study. However, an alignment that included *idh* gene sequences of all known patulin-producers including those in *Aspergillus*, *Byssochlamys* and *Paecilomyces* revealed a high degree of dissimilarity between these genera and *Penicillium* spp. Therefore, primer design was based exclusively on *Penicillium* sequences since they represent the majority of patulin-producers (Puel et al., 2010). In order to expand the range of detected species in future studies, separate primers may be targeted to non-*Penicillium* patulin-producers and combined in a multiplex LAMP assay as described for trichothecene-producers in *Fusarium* by Denschlag et al. (2014).

The LAMP assay with neutral red as indicator dye showed adequate results when incubated at 65 °C, with a color change clearly visible by the naked eye in positive samples. According to the manufacturer, the *Bst* polymerase used in the current assay has an optimum temperature range between 60 °C to 65 °C. This optimum temperature was fully matched by the chosen assay temperature. Although the temperature gradient revealed positive results also for lower incubation temperatures for the current LAMP assay, too low temperatures must be avoided because of the risk of non-specific binding of primers to DNA and therefore unspecific results. For monitoring of results, neutral red as pH-sensitive dye was used. A successful application of LAMP depends on the method selected for monitoring the reactions. Originally, they were detected by agarose gel electrophoresis resulting in a ladder-like pattern (Notomi, 2000). As this method is very time-consuming and cross contaminations are unavoidable due to the high amount of synthesized LAMP products, different in-tube detection methods were subsequently developed. One method is the detection of protons released during LAMP (Tanner et al., 2015). Using low concentrated buffers, the pH changes to acidic which can be detected by pH-sensitive indicator dyes such as neutral red. This color change from orange to pink was detectable in-tube after 60 min confirming the easy and fast detection of DNA by use of the new LAMP assay with neutral red.

The specificity of the developed LAMP assay was demonstrated to be very high for the detection of patulin-producing *Penicillium* spp. Both addition of purified DNA and direct addition of fungal conidia led to positive results for all available and tested *Penicillium* spp. that had been reported as patulin-producers in the literature (Frisvad et al., 2004; Puel et al., 2010). A positive result also occurred for *P. roqueforti* TMW 4.1599 and *P. coprophilum* TMW 4.2815. Paterson et al. (2003) found that *P. roqueforti* is positive for the *idh* gene, which explains a positive result in the LAMP assay. However, the ability of *P. roqueforti* to produce patulin is controversially discussed in literature. Production was confirmed by older studies (Paterson et al., 2003) but current research rather identifies the species as a non-producer of patulin (Frisvad, 2018; Puel et al., 2010). According to Nielsen et al. (2017), *P. roqueforti* contains most of the gene cluster needed for patulin production but misses some genes and is not able to produce patulin. It is assumed that older studies about patulin production of *P. roqueforti* were misleading because of confusion of analyzed strains with the closely related species *P. carneum* and *P. paneum*, both of which can produce patulin (Frisvad, 2018). Additionally, the result of thin-layer chromatography (TLC) analyses during the current study (results not shown) showed no patulin production in the *P. roqueforti* isolate available to us. As *P. roqueforti* mainly occurs on blue cheese, moldy baker's yeast, soil and wood and spoils rye bread and silage (Frisvad and Samson, 2004), false-positive results in tested fruits can be supposed to appear very rarely. TLC analyses of the *P. coprophilum* isolate also showed no patulin production which is in accordance to other studies indicating *P. coprophilum* as a non-patulin-producer (Frisvad and Samson, 2004; Houbraken et al., 2016). Nielsen et al. (2017) found that the intermediate isoepoxydon of the patulin biosynthetic pathway can be synthesized by *P. coprophilum* and that this species has partial patulin biosynthetic gene clusters. These findings can explain a positive reaction in the new LAMP assay. However, since *P. coprophilum* primarily occurs in animal dung, cereals and pet food (Frisvad and Samson, 2004; Houbraken et al., 2016; Samson and Pitt, 1990), false-positive results in tested fruits can be assumed to hardly appear. To the best of our knowledge, only Felšöciová et al. (2015) found one *P. coprophilum* isolate on Slovak wine grape samples taken from 2011 to 2013 among 251 *Penicillium* spp. *A. clavatus* strains were negative in the LAMP assay although they are patulin-producers. This is because the primers were designed specifically for *Penicillium* spp. Nevertheless, positive LAMP signals resulted also with DNA of the patulin-producers *Byssochlamys nivea* and *Paecilomyces saturatus*. Their *idh* genes are detectable by the designed primers even if their *idh* gene sequence showed considerable differences when compared to the *idh* genes of *Penicillium* spp. In summary, the high specificity of the developed assay was confirmed by testing 174 fungal strains representing 31 genera. Not all patulin-producing *Penicillium* spp. known from literature were available to the current study and they should be tested in further studies to acquire the full spectrum of detected species for this assay. The taxonomical identity of patulin-producing species is still under discussion and latest research has extended the number of patulin-producing species from 18 to 25 (Frisvad, 2018). These additional species should also be tested in

further studies.

For confirmation of the specificity of the LAMP assay, the nucleotide sequence of the smallest LAMP product of *P. expansum* TMW 4.2808 was sequenced. According to the NCBI database, the sequencing results showed 96% identity of the obtained sequence with the *idh* gene of different *P. expansum* strains (NRRL 35695, NRRL 6069, NRRL 35231, NRRL 2304, NRRL 35259, NRRL 32293, NRRL 32289). These results reveal the identity of the amplification products with the *idh* gene of the patulin biosynthetic gene cluster. The fact that no 100% identity was found can be explained by the application of direct sequencing of a rather small amplification product as well as by missing of a proof-reading function in the *Bst* polymerase which will result in a small number of sequence errors in the LAMP product used for sequencing template production.

The activity of loop primers during DNA amplification was tested in a real-time fluorimeter using the intercalating fluorescent dye V13-01184 for signal generation. In general, the LAMP reactions with loop primers showed an onset of DNA amplification that started 26 min earlier as compared to a reaction performed without any loop primers. The current study showed that loop primers do not contribute equally to the reaction speed, since the backward loop primer led to an earlier start of amplification than the forward primer when either was used separately in a LAMP reaction.

The developed LAMP assay revealed a sensitivity of at least 2.5 pg of purified DNA of *P. expansum* TMW 4.2808 per reaction. According to the NCBI database, the genome of *P. expansum* (MD-8) has a size of 32.35 Mbp and thus the minimum detected amount of DNA corresponds to 71.6 genome copies. In comparison to PCR-based assays for patulin-producers with detection limits for purified DNA of 0.5 ng (Luque et al., 2011) and 0.1 ng (Tannous et al., 2015), the developed LAMP assay has a 200- and 40-fold higher sensitivity, respectively. Moreover, the developed LAMP assay also detected its target when fungal spores were directly added to the reaction without any previous sample preparation and when a spore suspension was used with a limit of detection of 1×10^3 conidia per reaction.

Furthermore, the developed assay was applied to investigate artificially contaminated grapes and apples. The LAMP assay was tested by using the supernatant from grapes and apples obtained by simple washing and cell disruption steps. The grape samples showed positive LAMP signals after two days and the apple samples were positive after four days after inoculation with *P. expansum* TMW 4.2808. Thus, the detection was possible before visually detectable mold symptoms occurred. These findings indicate that a high simplification of sample preparation together with LAMP-based detection facilitate the assessment of samples for patulin-producing species. Hence, with this simple protocol, grapes harvested in Germany in September 2018 were tested as well. All tested grapes of different varieties and locations in Germany were negative in the LAMP assay. A possible explanation for this finding is the hot and dry summer of 2018 in Germany, with an average precipitation of only 130 l/m^2. Wine growing regions such as Hesse (Geisenheim, 90 l/m^2), Rhineland-Palatinate (Selzen, 125 l/m^2) or Baden-Wuerttemberg (Baden, 160 l/m^2) (Deutscher Wetterdienst, 2018) were especially short of rain fall. In dry summers, infestation of grapes with filamentous fungi is in general considerably lower as compared to normal or wet years. This fact may have caused the negative results found in the new LAMP assay. Moreover, apple juice, apple puree and grape juice were tested with the new LAMP assay (results not shown) to assess the possibilities of application of the assay. It was not possible to use undiluted juice, as the liquid was too acidic and the reaction mix with the pH-sensitive dye neutral red changed its color right after adding the juice sample and already before incubation at 65 °C. However, using 1:100 dilutions of these three matrices, the LAMP assay could be applied and revealed positive results when testing artificially contaminated juices and purees. Under these conditions, uninoculated controls gave negative results in all tested matrices.

In conclusion, the results of the current study show the general potential and applicability of the developed assay as a rapid and user-friendly tool for the detection of patulin-producing *Penicillium* species as well as *Byssochlamys nivea* and *Paecilomyces saturatus*. A specific and sensitive detection can be performed within 60 min of incubation and results can be monitored by naked eye inspection at day light. A simple sample preparation protocol and the use of simple equipment like a water bath or simple heating block make it a promising and economically affordable tool for on-site detection and quality control in the food and beverage industry as well as in agriculture. In its current form, the assay can be applied mainly by central surveillance laboratories. However, ready-to-use LAMP assays could be prepared in a freeze-dried format that could be provided in a kit for application in the field or in small scale laboratories. Besides the assay of Tone et al. (2017) that detects *P. expansum* among other non-patulin-producing species, no LAMP assay has been developed so far for the detection of patulin-producing species emphasizing the importance of a group-specific assay like the one developed in the current study.

Declarations of interest

The authors declare no conflict of interest.

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