Rapid and Sensitive Detection of the Main Contaminating Fungus *Penicillium restrictum* in Jet Fuel Using Loop-mediated Isothermal Amplification Combined with a Lateral Flow Dipstick

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Abstract: *We report a new contaminating fungus of jet fuel, Penicillium restrictum, which accounted for nearly 17% of the total sequence identified from five jet fuel samples as determined by the application of Illumina MiSeq sequencing-by-synthesis. We also report the development and validation of a new loop-mediated isothermal amplification (LAMP) assay combined with a lateral flow dipstick (LFD) for the repaid detection of P. restrictum. The optimal reaction conditions and primer set for LAMP were determined using a real-time turbidimeter. The LAMP-LFD assay was 1000-fold more sensitive than traditional PCR. P. restrictum could be detected specifically using the LAMP-LFD assay, and no amplification was observed when genomic DNA from another seven fungi found in jet fuel was tested. Eleven jet fuel samples from the field were tested using the LAMP-LFD assay we developed. Seven of them were positive for the presence of P. restrictum. These results were verified by traditional microbiological detection methods. Our results indicate that the LAMP-LFD assay is a rapid, accurate and sensitive tool for the detection of P. restrictum and could represent a new template for the detection of contaminating fungi in jet fuel.*

Keywords: *Illumina MiSeq sequencing-by-synthesis, Fungal contamination, Lateral flow dipstick, Loop-mediated isothermal amplification, Penicillium restrictum.*

Introduction

Microbial contamination accelerates deterioration of jet fuel, decreases fuel efficiency and stability, and corrodes the fuel system and infrastructure [15, 21]. The presence of filamentous fungi in fuel may lead to the formation of suspended substances and thick fugal mats that cause automatic gauges to malfunction due to physical blockage of pipelines and filters. The accumulation of such fungal structures around the fuel tank probe may result in fuel gauge malfunction, which threatens flight safety [22, 25].

Here, by the application of next-generation DNA sequencing (NGS) techniques, we identify a new contaminating fungus in jet fuel, *Penicillium restrictum*. *P. restrictum* is highly adaptable and widely distributed; it has been isolated from soil, air, water, food, etc., but it has not previously been recovered from jet fuel [2, 11, 19]. *P. restrictum* has stimulated widespread interest because of its ability to produce lipases, which are crucial enzymes used in medicines,

clinical reagents and the food industry [3, 10]. However, Vesper [26] found that some species of *P. restrictum* could produce hemolysins, which is one of the main elements of sick building syndrome. Thus, *P. restrictum* as a contaminating fungus may not only reduce the quality of jet fuel, but is also a potential threat to the health of depot staffs.

Traditional methods for the identification of microbial contamination based on biology and morphology require the cultivation of microorganisms [5, 8, 9]. However, Sharkey et al. [24] reported that only 1% of microbes that have been identified can actually be cultivated in the laboratory. The limitations of traditional methods led to the development of PCR-based methods for microbial identification. Denaro et al. [6] recovered and identified microbial contaminants in JP-8 jet fuel samples by the method of traditional culture and PCR methods. They identified 36 operational taxonomic units (OTUs) of which 28 had never been described previously. Rauch et al. [22] identified Aureobasidium pullulans and Discophaerina fagi from fuel samples of United States Air Force aviation fuel tanks by 18S rRNA gene sequencing based on PCR method. Meanwhile, Discophaerina fagi as fungal contaminants has never been reported before. However, PCR methods are time consuming and also require highly trained operators and expensive equipment. The main means of detecting *P. restrictum* are presently the traditional and PCR methods [5, 14, 23] whose advantages and limitations have been elaborated above.

Loop-mediated isothermal amplification (LAMP) assay is a rapid and specific DNA amplification method described originally by Notomi et al. [20]. A set of four primers were used in the LAMP reaction which could correctly hybridize to the target sequence that results in the high specificity of LAMP. Compared with traditional and PCR methods, the LAMP method does not require special and/or expensive equipment or a rigorous experimental environment. Thus, the LAMP method has been applied in many areas, such as the detection of viruses, bacteria, fungi and genetically modified food ingredients [1, 7, 13, 30]. The application of a lateral flow dipstick has been shown to improve the rapid and specific detection of LAMP products [4, 27]. The LAMP amplification product with biotin-labelled was hybridized with fluorescein isothiocyanate (FITC) hybridization that could be detected by LFD. The test line of LFD will show reddish-purple when the amplicon was successfully trapped by streptavidin. This combination can visualize the LAMP product in minutes without special instrumentation and this is a crucial advantage of the LAMP-LFD method in field applications.

In order to identify the main contaminating fungus in jet fuel, the next-generation DNA sequencing (NGS) techniques has been taken application in this study. Meanwhile, this study develops a rapid new method for detecting *P. restrictum*, one of the main contaminating fungi of jet fuel, using loop-mediated isothermal amplification combined with a lateral flow dipstick, and then it was used in the field for early detection.

Materials and methods

Samples for Illumina MiSeq sequencing-by-synthesis

We collected five jet fuel samples from one army oil deposit in southwest China, named A, B, C, D and E, respectively. The sampling locations were all at the bottom of fuel storage tanks. All samples were sent to the laboratory within 24 h.

Fungal samples and cultivation

Eight fungi, *P. stickii* (CGMCC 3.8217), *P. digitatum* （CGMCC 3.5931), *P. restrictum* (CGMCC 3.8261), *A. penicillioides* (CGMCC 3.3978), *Trichoderma viride* (CGMCC

3.6619), *Fusarium oxysporum* (CGMCC 3.6809), *Khuskia oryzae* (CGMCC 3.8851), and *Amorphotheca resinae* which were separated and identified from jet fuel by our research group were used in this study. All the fungi expect *Amorphotheca resinae*were provided by the China General Microbiological Culture Collection Center and were cultured in Sabouraud liquid medium at 25 °C for 4 d.

DNA extraction

Five jet fuel samples were respectively filtered through 0.25 μm membranes, then the membranes were repeatedly flushed with 1.5 ml ultrapure water which was collected in 2 ml Eppendorf tubes, respectively. Microbial DNA was extracted from the respective samples using the General Genomic DNA Extraction Kit (Takara Biotechnoloy Co., Ltd., Dalian, China) according to the manufacturer's instructions, and then microbial DNA was sent to Beijing Novogene Bioinformatics Technology Co., Ltd. for Illumina MiSeq sequencing-by-synthesis. Total DNA from eight fungal strains was extracted as above and the concentrations of DNA were determined by spectrophotometer (Thermo Scientific NanoDrop 2000C). The extracted DNA of eight fungal strains was stored at −20 °C until use.

Primer design for LAMP

Three sets of primers were designed using Primer Explorer V4 software (http://primerexplorer.jp/e/) according to the published conserved internal transcribed spacer (ITS) sequences of *P. restrictum* (GenBank accession no. AF033459.1). Each set of primers consisted of two outer primers (F3, P-B3), a backward inner primer (BIP), a forward inner primer (FIP), and two loop primers (LF, LB). In all sets of primers, the 5′ end of FIP and LF was labeled with biotin and fluorescein isothiocyanate (FITC) respectively as probes. Table 1 shows the primer sequences.

Table 1. Three sets of primers used for LAMP assay

Primer	Sequence（5′—3′）
P1-F3	AGGGATACCCGCTGAACTT
P1-B3	CTCGTTGAAGGAGCTTCACA
P1-FIP	TGAGCTCTTGCCGCTTCACTC-GCGGAGGAAAAGAAACCAAC
P1-BIP	TTGCAGAGGATGCTTCGGGAG-TCCCATACGGGATTCTCACC
P1-LF	GCCGTTACTGGGGCAATCC
P1-LB	GCCCCCATCTAAGTGCTCTGG
P2-F3	AGAAACCAACAGGGATTGCC
P2-B3	TCGACTCGTTGAAGGAGCT
P2-FIP	GCAAATTACAATGCGGACCCCG-GTAACGGCGAGTGAAGCG
P2-BIP	GGAGTGGCCCCCATCTAAGTG-ACACCCCATCCCATACGG
P2-LF	GGGCCAGCTTTCAAATTTGAGCT
P2-LB	GGCCGTCATAGAGGGTGAGAA
P3-F3	AGGGATACCCGCTGAACTT
P3-B3	TCCCATACGGGATTCTCACC
P3-FIP	TTGAGCTCTTGCCGCTTCACTC- GCGGAGGAAAAGAAACCAAC
P3-BIP	ATTTGCAGAGGATGCTTCGGGA-TCTATGACGGCCCGTTCC
P3-LF	GCCGTTACTGGGGCAATC
P3-LB	GGCCCCCATCTAAGTGCT

LAMP reaction and optimization of reaction conditions and primers

The LAMP reaction was modified compared with that by Notomi et al. [20] in that the reaction volume was 25 µl, including 1.6 µM of each of primers BIP and FIP, 0.2 µM of each of primers F3 and B3, 0.8 µM of each of primers LF and LB, 0.8 M betaine (Sigma-Aldrich, St. Louis, MO), 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 8 mM $MgSO_4$, 0.1% Tween-20, 10 mM $(NH4)_2SO_4$, 8 U of *Bst* DNA polymerase, 1.4 µM of each dNTP and 2 µl of template DNA. Reaction were carried out at 61 °C, 63 °C or 65 °C for 60 min. Ultrapure water was added instead of DNA as a negative control. The LAMP reaction was monitored using a real-time turbidimeter (Loopamp LA-320, Japan). From the amplification curve of the LAMP reaction obtained from the real-time turbidimeter, we could determine the best reaction conditions and primers, based on the amplification efficiency and threshold time.

Sensitivity of the LAMP assay

The sensitivity of the LAMP-LFD method for detection of *P. restrictum* was compared with that of PCR. Genomic DNA of *P. restrictum* was serially 10-fold-diluted from 10^1 to 10^8 with the original concentration being 42 ng/µl. The diluted genomic DNA was used as DNA template to determine the analytical sensitivity of LAMP reactions and PCR. PCR was performed with 35 cycles in a 50 µl volume, including 20 µM each of premiers F3 and B3, 5 UEx *Taq*DNA polymerase (Takara), 2.5 µl 10×buffer, 2.5 mM dNTPs and 2 µl DNA template. The PCR conditions were as follows: 5 min at 94 °C; 35 cycles of 40 s at 95 °C, 40 s at 52 °C, and 90 s at 72 °C; with a final extension at 72 °C for 10 min. Ultrapure water was added instead of DNA in the negative control. The PCR products were analyzed by 1% agarose gel electrophoresis, and the LAMP reaction was visualized by real-time turbidimeter and LFD.

Stability of LAMP-LFD assay

Genomic DNA of *P. restrictum* was serially 10-fold-diluted, then DNA at the detection limit determined above was used as the temple for LAMP reactions. The reaction was performed with three samples in parallel, and ultrapure water was added to the negative control instead of DNA. The LAMP reaction was visualized by real-time turbidimeter and LFD.

Specificity of the LAMP assay

The specificity of the LAMP and PCR assays was analyzed by using DNA templates extracted as above from pure cultures of eight fungal strains. Ultrapure water was used instead of DNA in the negative control. PCR products were analyzed by 1% agarose gel electrophoresis, and the LAMP reaction was visualized by real-time turbidimeter and LFD.

Application of LAMP-LFD assay to field jet fuel samples

We collected 11 jet fuel samples from different tanks, numbered J-1, J-2, J-3, J-4, J-5, J-6, J-7, J-8, J-9, J-10 and J-11. Genomic DNA was extracted according to the described above method then prepared as templates for the application of the LAMP-LFD assay. Ultrapure water was used instead of DNA in the negative control. To verify the feasibility and accuracy of the LAMP-LFD assay, we undertook fungal culture experiments on the 11 jet fuel samples using Czapek-Dox medium with added streptomycin and kanamycin (0.3 g per 100 ml). Identification of *P. restrictum* was based on morphological and physiological characteristics [16, 28].

Results

Illumina MiSeq sequencing-by-synthesis

In order to determine fungal contamination in jet fuel, five jet fuel samples collected from an army oil deposit in southeast of china were analyzed through Illumina MiSeq sequencing-by-synthesis. Detailed sequencing reports were generated by a commercial supplier, Beijing Novogene Bioinformatics Technology Co., Ltd. According to their data obtained, *P. restrictum,* resulted the main fungal contaminant which had not been reported before.

Rarefaction curve

Rarefaction curves can directly reflect the coverage of sequencing data, and indirectly reflect the richness of species in the sample. The rarefaction curves from five jet fuel samples (97% similarity) approached an asymptote, indicating that the sequencing data represented sufficient coverage of all the microbial communities present in the samples (Fig. 1).

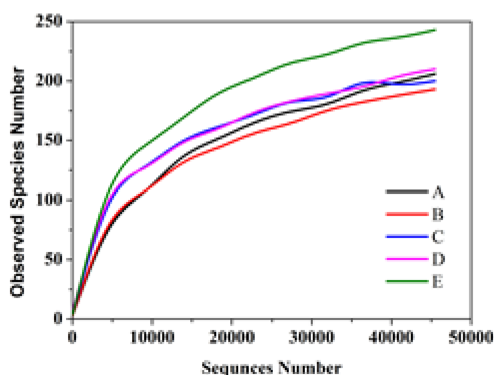

Fig. 1. Rarefaction curves for five jet fuel samples

Relative abundance of species

From the sequencing report, we obtained the relative abundance of each species in the jet fuel samples. At the genus level, *Penicillium* were the second main contaminating fungi after *Amorphotheca* spp. (Fig. 2A). At the species level, *P. restrictum* accounted for 99.01% of the *Penicillium* (Fig. 2B), showing that it was one of the main contaminating fungi in jet fuel.

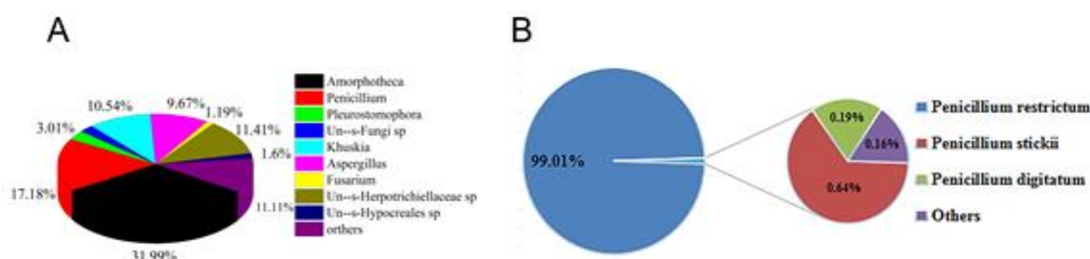

Fig. 2 Relative abundances of the dominant contaminating fungi in jet fuel samples at the level of genus (A) and species (B)

LAMP-LFD assay

Optimization of reaction conditions and primer set for the LAMP assay

The LAMP reaction was carried out in isothermal conditions for 60 min in the range 61 °C to 65 °C using three respective sets of primers with the genomic DNA of *P. restrictum* as the template. The amplification efficiency of the LAMP reaction at 61 °C (Fig.3 A) was significantly lower than that at 63 °C (Fig. 3B) and 65 °C (Fig. 3C). Comparing the amplification efficiency of the LAMP reaction using different sets of primers at 63 and 65 °C,

the best results were obtained from primer set-P3 at 63 °C. Therefore, the optimum reactions conditions for the LAMP assay were 63 °C for 60 min using primer set-P3.

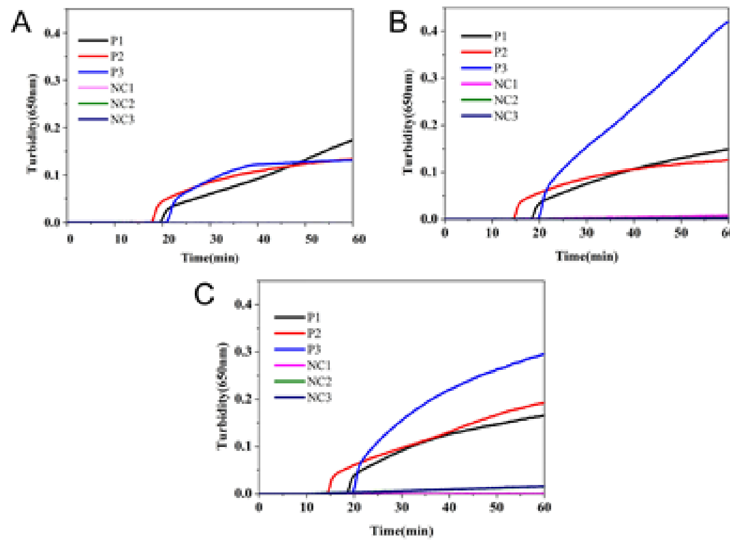

Fig. 3 LAMP results with three primer sets,
P1, P2, and P3, at 61 °C (A), 63 °C (B) and 65 °C (C)

Comparison of the sensitivity of LAMP-LFD and PCR assays

The detection sensitivity of LAMP-LFD was compared to that of PCR by using different dilutions of genomic *P. restrictum* DNA as the DNA template. The detection limit of the PCR assay was 4.2 pg/μl (Fig. 4B). The detection limit of the LAMP assay was 4.2 fg/μl. This value was consistent whether visualized using a real-time turbidimeter or LFD (Fig. 4A and Fig. 4C). Thus, the LAMP-LFD assay was 1000-times more sensitive than the PCR method.

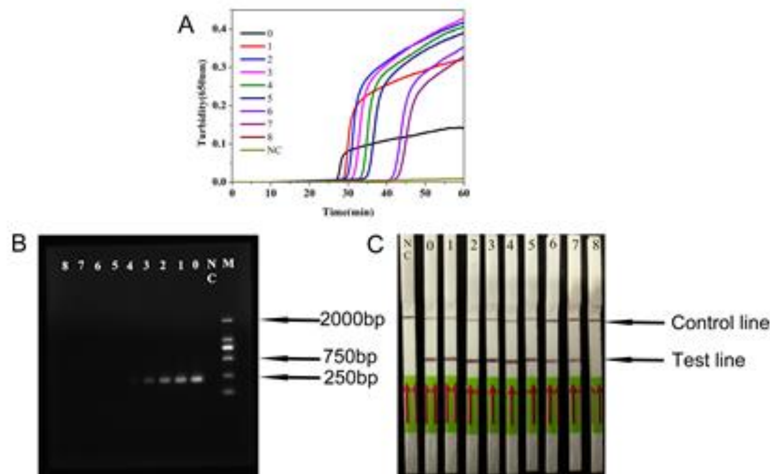

Fig. 4 Sensitivity of LAMP (A), PCR (B) and LAMP-LFD (C) assays:
M: DNA marker; NC: negative control (water as template); 0-8: different dilutions of genomic DNA of *P. restrictum* (10^0-10^8, corresponding to 42 ng/μl, 4.2 ng/μl, 420 pg/μl, 42 pg/μl, 4.2 pg/μl, 420 fg/μl, 42 fg/μl, 4.2 fg/μl, and 0.42 fg/μl).

Stability of LAMP-LFD assay

To analyze the stability of the LAMP-LFD assay, we used *P. restrictum* DNA diluted 107-fold (to 4.2 fg/μl) as a template. Three replicates with such DNA resulted in positive LAMP reactions visualized by real-time turbidimeter (Fig. 5A) and LFD (Fig. 5B).

The negative control samples gave no amplification. These results indicate that the LAMP-LFD assay has good stability.

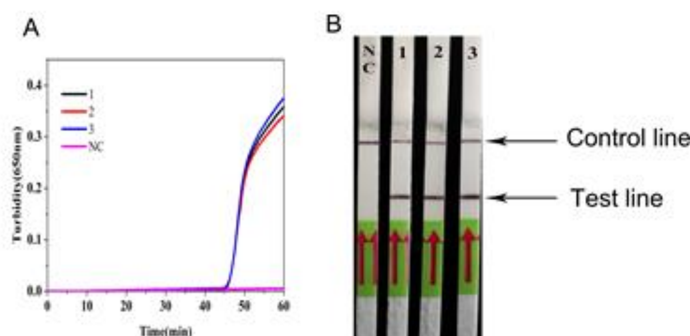

Fig. 5 Stability of the LAMP-LFD assay determined by real-time turbidimeter (A) and LFD (B); 1, 2 and 3 were the three samples tested in parallel.

Comparison of the specificity of the LAMP-LFD and PCR assays

The specificity of the LAMP-LFD assay was evaluated using the genomic DNA of eight fungal strainsthat were present in jet fuel according to the results of Illumina MiSeq sequencing-by-synthesis. PCR was used as a control. Only the LAMP reaction with *P. restrictum* DNA as the template gave a positive result (Fig. 6A and Fig. 6C). In contrast, significant non-specific amplification was observed in PCR with *P. stickii* and *Amorphotheca resinae* DNA (Fig. 6B).This indicted that the specificity of the LAMP-LFD assay in detecting *P. restrictum* is better than that of the PCR method.

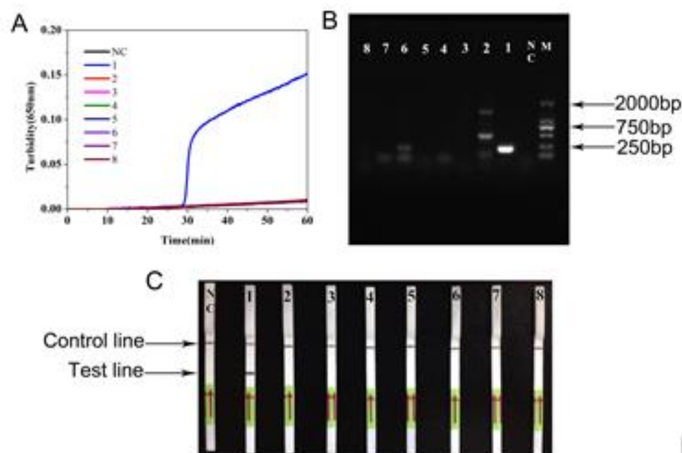

Fig. 6. Specificity of the LAMP (A), PCR (B) and LAMP-LFD (C) assays: M: DNA marker; 1-8: *P. restrictum*, *P. stickii*, *P. digitatum*, *Aspergillus penicillioides*, *Khuskia oryzae*, *Amorphotheca resinae*, *Trichoderma viride*, *Fusarium oxysporum*.

Applicability of the LAMP-LFD assay for field detection

The applicability of the LAMP-LFD assay in field conditions was evaluated by testing 11 jet fuel samples that collected from an army oil deposit. Seven of the 11 samples were positive for *P. restrictum* on application of the LAMP-LFD assay, and no amplification occurred in the negative control reaction (Fig. 7A). These data were consistent with the detection of *P. restrictum* by traditional (microbiological) methods (Fig. 7B) and indicate that the LAMP-LFD assay is suitable for application in field detection of *P. restrictum.*

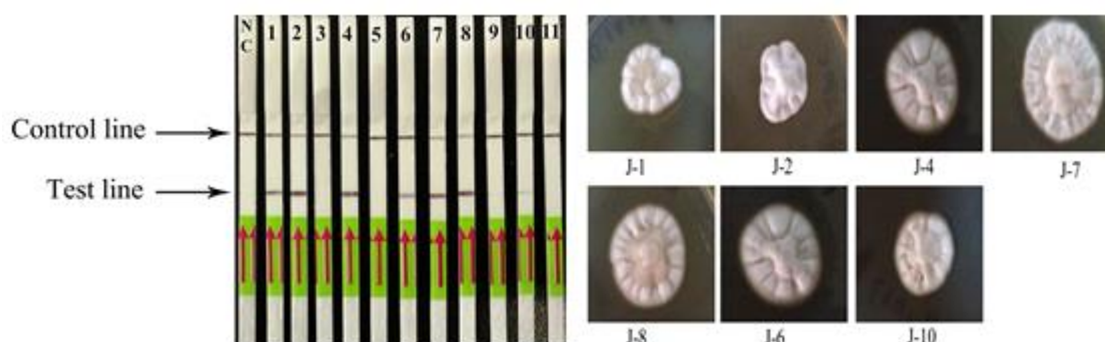

Fig. 7 Field detection of *P. restrictum* in jet fuel samples
using the LAMP-LFD assay (A) and traditional microbiological methods (B)

Discussion

Microbial contamination of jet fuel may represent a serious concern, due to a progressive loss of chemical potential energy, indoor contamination, and threats to flight safety. Cultivation-based and PCR methods have been widely used to detect fungal contamination in jet fuels, but these methods have inherent flaws since they are time-consuming, and/or require expensive equipment, and/or have poor specificity and sensitivity. This makes difficult the application of such protocols in a number of laboratories as well as in field tests.

Gaylarde et al. [12] has summarized previous work and citied the problem caused by microbial contamination which problems caused fungal contamination no less than bacteria. Therefore, it has a certain significance for the study of fungal contamination in jet fuel. Meanwhile, as strategic jet fuel reserve, it could not solve the problem of microbial contamination simply using fungicides recommended by the International Air Transport Association. The main reason is that Kathon FP1.5 and Biobor JF as the only two microbicides approved for jet fuel treatment do not accepted by Air Force of many countries, such as china. In order to ensure the safety of strategic jet fuel reserves, many fuel tanks were underground tanks and cave tanks. These tanks has been built for a long time, and the breathing valve of most cave tanks interconnected and collect to the main breathing valve which extends outside the cave. Thus, fugal contamination of one tank could easily lead to a cross-infection. Therefore, it required to develop a repaid and accurate method to detection of microbial contamination, and a repaid treatment should be carried out to prevent contamination expansion.

To identify and detect *P. restrictum*, which is reported here as one of the main fungal contaminants in jet fuels, we perfected and evaluated a new LAMP-LFD assay. This latter combines the advantages of the LAMP assay in specifically and efficiently amplifying the ITS DNA of *P. restrictum* with the ease of LFD in visualizing the results within a few minutes. The LAMP reaction was carried out at 63 °C for 60 min, which saved 1.5 h compared with PCR (including the detection step). The detection limit of genomic DNA of *P. restrictum* by LAMP-LFD was 4.2 fg/μl while the PCR limit was 4.2 pg/μl. Thus the LAMP-LFD assay was 1000-times more sensitive than the PCR method. There was no cross amplification in LAMP-LFD from other fungal species that we found to be present in jet fuel by Illumina MiSeq sequencing-by-synthesis. On the other hand, the PCR assay showed poor specificity. The results of *P. restrictum* detection in field jet fuel samples by the LAMP-LFD assay were consistent with the results obtained by traditional microbiological methods, verifying that the LAMP-LFD assay is practical for field detection.

For the perfection of the method, a real-time turbidimeter was used as the detector in the LAMP reaction. The main advantages of this approach over gel electrophoresis are ease of use and real-time monitoring of the reaction, which is not possible by gel electrophoresis. The application of the real-time turbidimeter also makes possible quantitative detection of *P. restrictum* by LAMP. For instance, Luo et al. [18] described a method of quantitative detection of *A. flavus* and *A. parasiticus* based on the linear relationship between the concentration of DNA and the threshold time of the real-time amplification curve.

The application of next-generation DNA sequencing techniques proved a breakthrough of microbial community analysis in jet fuels. NGS techniques have overcome the limits of cultivation-based and PCR-based methods demonstrating to be a reliable first choice for complex microbial community analysis [17, 29]. By using NGS, we could determine the variation in the microbial community and the microbial characteristics in jet fuel in different stages of production and use. Therefore, identification of both nature and degree of microbial contamination in jet fuels can allow the adoption of an adequate contrast to the deterioration processes. Through the High-throughput sequencing process, one fungal's relative abundance could be analyzed based on the number of DNA of this fugal that detected. Thus, high-throughput sequencing has the quantitative function. Therefore, the relative abundance in some extent can be reflected the proportion of a fungal in the whole fungal contamination biomass. As evidenced in this study, the possible combination of NGS with LAMP-LFD assay deserves attention for its broad application potentialities in the prevention and control of microbial spoilage of jet fuels.

The ultimate aim of us was to develop a LAMP integrated microfluidic chip (on-chip LAMP) for multiplex detection of fungal contamination in jet fuel. In order to achieve that goal, we have united Laboratory of Biochemistry and Molecular Biology of Ningbo University and CapitalBio Co., China to develop a new kind of microfluidic chip and it matching equipment. It has been successfully applied to detection of pathogenic bacteria in aquatic animals [31]. Therefore, the LAMP-LFD method developed in this study for rapid detection of *P. restrictum* as a template could widely generalized to other fungal contamination which lay the foundation for the rapid detection of various fungal contamination with on-chip LAMP.

Conclusion

By the application of next-generation DNA sequencing techniques, a new fungal contaminant of jet fuels, *P. restrictum*, was identified that had not been reported before. In this paper, a highly specific, simple, sensitive and rapid LAMP-LFD assay for the detection of *P. restrictum* in jet fuels is described. A preliminarily validation for its application in routine field analyses has been performed. It is worth noting that the LAMP-LFD is 1000 times sensitive than PCR-based methods. Therefore these results indicate that the LAMP-LFD assay can be taken into account as a new paradigm for the detection of microbial contamination in jet fuels.

Acknowledgements

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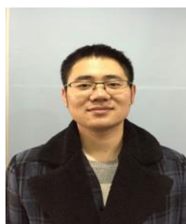

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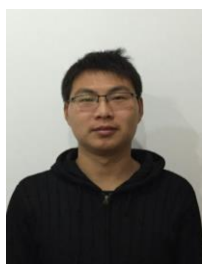

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