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Repaid and Accurate Detection of *Khuskia oryzae* by Loop-mediated isothermal Amplification Combined with a Lateral Flow Dipstick

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Khuskia oryzae being as a contaminating fungus of jet fuel results in many damage. We have developed and valuated a repaid and accurate method for detection of *Khuskia oryzae* by loop- mediated isothermal amplification (LAMP) combined with a lateral flow dipstick (LFD).The result could be visualized by LFD within 10 min and did not rely on expensive experiment instrumentation. The detection limit of DNA of *Khuskia oryzae* by LAMP-LFD was 230fg/ul which was 10-fold more sensitive than LAMP and PCR methods.*Khuskia oryzae* could be specifically detected by LAMP-LFD, and no positive result could be observed when another 5 control genomic DNA were used. The whole process of detection by LAMP-LFD was 80 min which saved 90min compared to PCR method. The LAMP-LFD assay developed by this study was used for field detection and successfully detected *Khuskia oryzae* from the jet fuel samples. The LAMP combined with LFD makes the detection of *Khuskia oryzae* to be more simple, repaid and accurate, therefore, it will be a new tool for field detection.

1. Introduction

Microbial contamination of jet fuel may cause great damage to the storage equipment and fuel system (Rauch et al 2006, Gaylarde 1991). Fungal contamination were the dominate reason for the biofilm existed in jet fuel which was the biggest threat to fly safe, for example ,the B-52 crash in 1958 (Rauch et al 2006)were directly caused by the plugging of an in-line fuel filter which attributed by the biofilm. In addition, the microbial contamination of jet fuel will not only result in huge economic losses, but also greatly increase the maintenance costs. Therefore, the microbial contamination of jet fuel has aroused widespread concern since 1960's.The main methods for detection of contaminating microorganism were traditional methods based on cultivation (Ferrari et al 1998) and methods based on PCR (Raikos et al 2011). The tradition methods need the living microorganisms and time-consuming. Meanwhile, Sharkey F.H (2004) has indicated that only one percent of microbial in the environment including jet fuel has the identified. The methods based on PCR has been widely used for detection contaminating microorganism in jet fuel (Raikos et al 2011, Brown et al 2010). However, PCR methods requires a high technical skill and expensive equipments.

Khuskia oryzae has been identified by the next-generation DNA sequencing (NGS) techniques which was used to analyze the microbiology diversity of the jet fuel.*Khuskia oryzae* accounts for 10.1% of contaminating fungi according to the result of NGS.*Khuskia oryzae* is an endophyte which could produce bioactive secondary metabolites and could be widely found in plant (Randa and Kamel 2013). The detection of *Khuskia oryzae* are also focused on tradition methods (Sempere and Santamarina 2006) and PCR methods (Silva et al 2008). The limitation of these methods for detection of *Khuskia oryzae* are the same as above.

Loop- mediated isothermal amplification (LAMP) is a new method for the amplification of DNA which has been widely used for detection of fungi, bacteria and virus (Mori et al 2013). The LAMP did not rely on special and professional instruments, even a thermostat water bath could cover the requirement. The product of LAMP reaction are commonly analyzed by agarose gel electrophoresis. However, this method also needs special

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instruments for agarose gel electrophoresis. In contrast, lateral flow dipstick (LFD) did not need any professional instruments and the process of detection just required 5-10 min compared to 45–60 min for gel electrophoresis (Wang 2013) .Therefor, the aim of this study is to develop a repaid and accurate methods for detection of *Khuskia oryzae* by the loop- mediated isothermal amplification (LAMP) combined a lateral flow dipstick (LFD).

2. Materials and Methods

2.1 Samples and DNA extraction

Total 6 fungi strains *Khuskia oryzae*, *Cladosporium resinae*,*Trichoderma viride*, *Aureobasidium pullulans*, *Penicllium restrictum* and *Aspergillus penicillioides* were used in this study .All fungi were provided by China General Microbiological Culture Collection Center (CGMCC) and cultivated at 25℃ in Potato Dextrose Agar medium for 4d.The DNA of fungi were abstracted by the the General Genomic DNA extraction kit (Takara Biotechnology Co., Ltd) according to the manufacturer's instructions. The extracted DNA were stored at -20℃until used.

2.2 Primer design

Two sets of primers targeting the ITS (GenBank: FN435719.1) of *Khuskia oryzae* were designed using the PrimerExplorer V4 software (Table 1). The forward inner primer (FIP) of each primer sets was labeled with biotin at 5 ' end and the same position of loop primer (LF) was labeled with FITC.

Table 1: Primers for detection of Khuskia oryzae used LAMP assay

Primer	Primer designation	Sequence(5ˈ-3ˈ)
K1-F3	Forward-outer primer	GGTTCTGGCATCGATGAAGA
K1-B3	Backward-outer prime	AGAGGACTACTGCCACTCC
K1-FIP	Forward-inner primer (F1c +TTTT +F2)	TGGGCGCAATGTGCGTTCAAACGCAGCGAAATGCGATA
K1-BIP	Backward-inner primer (B1c +TTTT +B2)	CTAGTGGGCATGCCTGTTCGATACGGAGGCCGTAGAGTC
K1-LF	Loop-forward primer	GATTCGATGATTCACTGAATTCTGC
K1-LB	Loop-backward primer	TCAACCCCTAAGCACAGCTTAC
K2-F3	Forward-outer primer	GGTTCTGGCATCGATGAAGA
K2-B3	Backward-outer prime	AGAGGACTACTGCCACTCC
K2-FIP	Forward-inner primer (F1c +TTTT +F2)	ATGGGCGCAATGTGCGTTCAACGCAGCGAAATGCGATA
K2-BIP	Backward-inner primer (B1c +TTTT +B2)	TTCGAGCGTCATTTCAACCCCTCATTAGGGAACTACGGAGGC
K2-LF	Loop-forward primer	GATTCGATGATTCACTGAATTCTGC
K2-LB	Loop-backward primer	AGCACAGCTTACTGTTGGGAC

2.3 LAMP reaction

The LAMP reaction was optimized comparing to Notomi(2000) that the reaction volume was 25μL, including 0.2 μM of each F3 and B3, 1.6 μM of each FIP and BIP, 0.8 μM of each LF and LB,0.8 M betaine, 10mM KCl,20mM Tris-HCl (PH 8.8) , 0.1% Tween 20,10 mM $(NH_4)_2$ SO_4,8mM $MgSO_4$,1.4mM dNTPs, 8U Bst DNA

and 2μL of template DNA. The negative control did not contain the template DNA which was replaced by ultrapure water.In order to determine the best reaction condition, the LAMP reaction was carried out at different temperature of 61,63 and 65℃ for 60min.The total LAMP reaction were monitored by Realtime Turbidimeter (Loopamp LA-320,Japan).

2.4 Sensitivity of LAMP assay

Ten-fold serial dilutions (10^0–10^{-8}) of DNA extracted from *Khuskia oryzae* were used as the template for LAMP reaction with the optimized condition described above. The LAMP reaction product was respectively detected and visualized by Realtime Turbidimeter (Loopamp LA-320, Japan) and LFD .In addition, the PCR method was also used as a control for LAMP-LFD. The PCR reaction were conducted in 50μl reaction mixture containing 2.5μl 10×buffer ,20μM of each K1-F3 and K1-B3,5U Ex Taq DNA polymerase(TaKaRa),2.5mM dNTPs and 3μl template of DNA. And the PCR reaction condition was as follows: 2 min at 94℃ ;1 min at 94°C ,1 min at 52°C , 2.5 minat 72°C, 35 cycles; 7.5 min at 72°C .The PCR reaction product was detected by electrophoresis on a 1 % agarose gel.

2.5 Specificity of LAMP assay

The specificity of LAMP-LFD compared to PCR was evaluated using the template DNA abstracted from the 6 fungi strains at the condition described above. The LAMP reaction product was respectively detected and visualized by Realtime Turbidimeter (Loopamp LA-320, Japan) and LFD, while, the PCR reaction product was analyzed by 1% agarose gel electrophoresis.

2.6 Application of LAMP-LFD for field detection

12 jet fuel samples were collected from one army oil depot, and numbered 1 to 12. The DNA of samples were abstracted as above, then taken as template for the LAMP -LFD assay.

3. Results

3.1 Optimize the LAMP reaction condition and primers set

The two sets of primers were applied for the LAMP reaction with the DNA of *Khuskia oryza*e at the condition of 61 (Fig.1 A), 63 (Fig,1 B) and 65℃ (Fig.1 C) for 60 min. The reaction were monitored by Realtime Turbidimeter. The LAMP reaction with primers set of K1 at 63℃ has the highest amplification efficiency and minimum threshold time (tm) compared to other reactions .Therefor, the best reaction condition of LAMP is 63℃ for 60min, and the optimum primers set is K1.

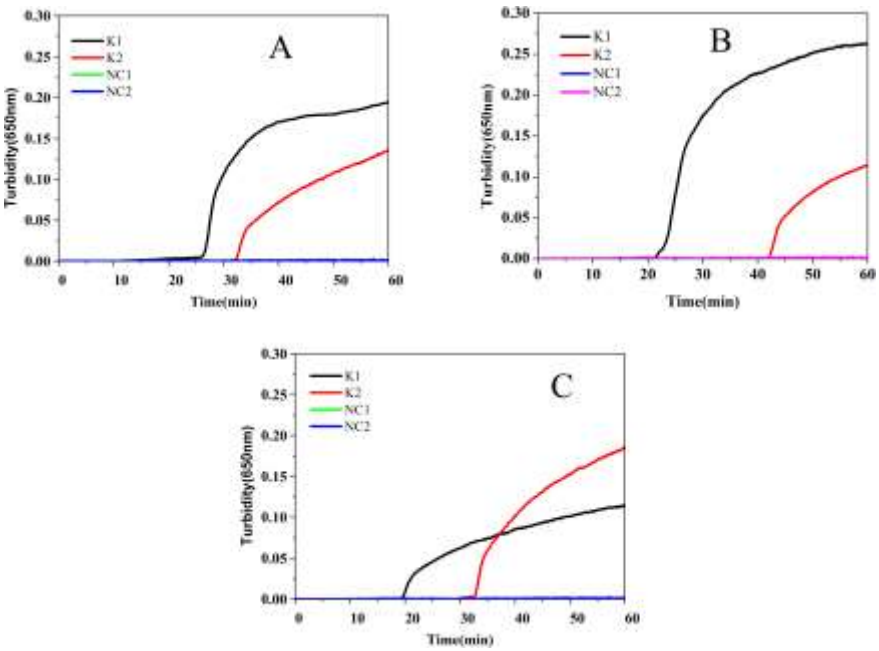

Figure 1: LAMP reaction with the two primers sets at different temperatures.

3.2 LAMP reaction condition and primers set

PCR, LAMP and LAMP-LFD reaction were carried out using the ten-fold serial dilutions of genomic DNA extracted from *Khuskia oryzae* as template. The LAMP assay could detected the template at 230fg/µl and 2.3pg/ µl respectively by LFD and Realtime Turbidimeter. The detection limit of PCR was 2.3pg/ µl .These results indicated that sensitivity of LAMP -LFD for detection of *Khuskia oryzae* was 10 time higher than LAMP and PCR.

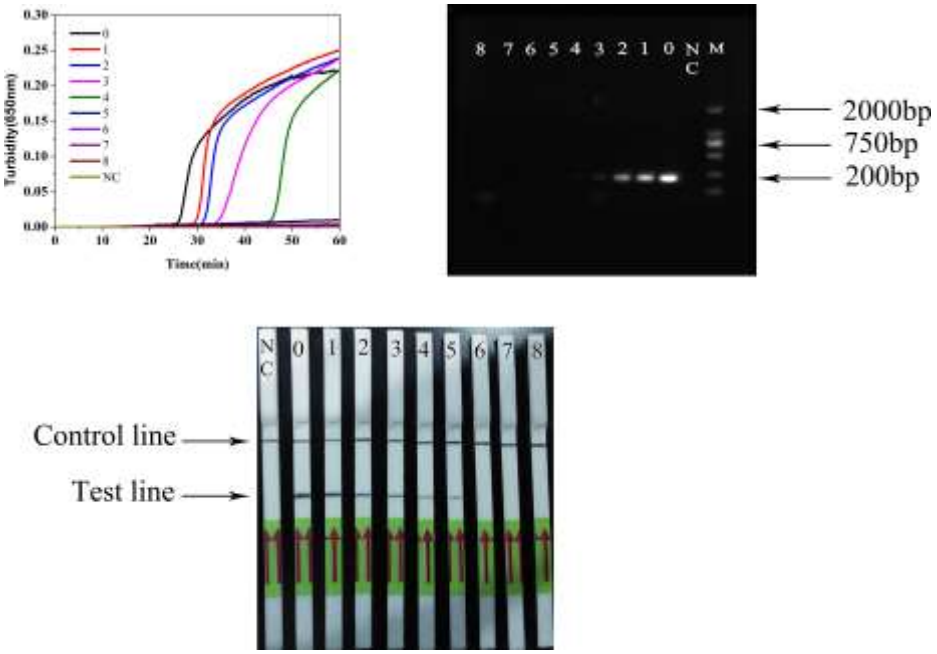

Figure 2: Sensitivity of LAMP-LFD (C) compared to LAMP (A) and PCR(B) for the detection of Khuskia oryzae genomic DNA.0-8: the serial dilutions of Khuskia oryzae genomic DNA from 10^0 to 10^{-8}(23ng/µl to 0.23fg/µl)

3.3 Comparison of specificity between PCR, LAMP and LAMP-LFD

The specificity of LAMP-LFD(Fig.3 C)for detection *Khuskia oryzae* was evaluated by comparing to LAMP (Fig.3 A) and PCR(Fig.3 B) using 5 fungi for control. The positive result of LAMP and LAMP-LFD were observed only when the template of *Khuskia oryzae* used, and the reactions with other template gave the negative results.

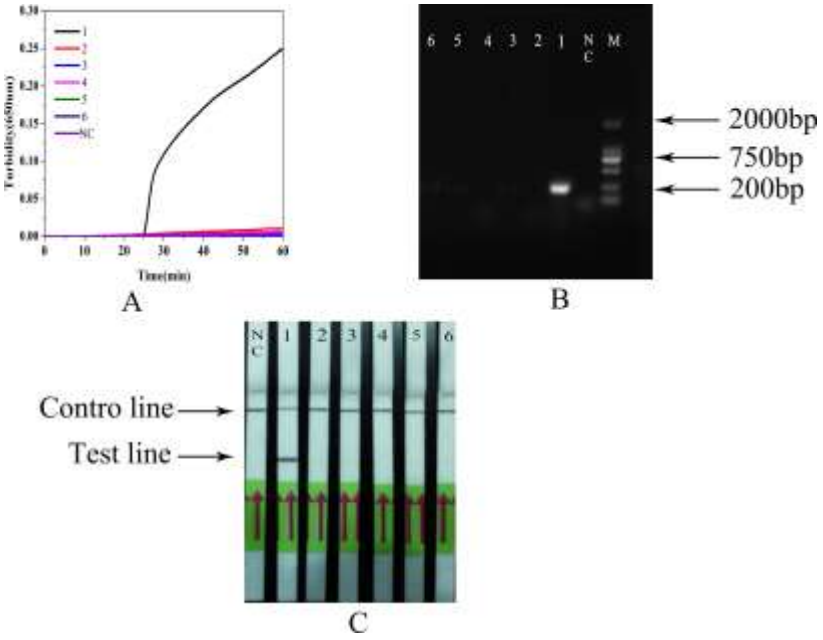

Figure 4: Specificity of LAMP-LFD (C)compared to LAMP (A)and PCR(B) for the detection of Khuskia oryzae genomic DNA .1-6: Khuskia oryzae, Cladosporium resinae, Trichoderma viride , Aureobasidium pullulans, Penicllium restrictum ,Aspergillus penicillioides.

3.4 *Khuskia oryzae* detection in jet fuel samples

12 jet fuel samples were used to test the application of LAMP-LFD assay for detection of *Khuskia oryzae* with field condition .The samples of 1, 2, 3, 6, 8, 10 and 11 were result in positive reaction, while, other of 12 samples and negative control have no amplification.

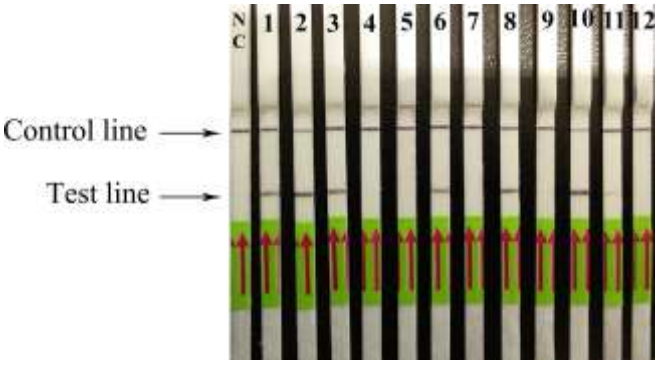

Figure 5: Detecion of Khuskia oryzae in jet fuel samples with the LAMP-LFD.

4. Conclusions

Microbial contamination, especially fungal contamination, of jet fuel has become a huge threat to storage and fly safe, and it will be more worse with the increase of storage time(Passman 2013).*Khuskia oryzae* has been identified from the jet fuel and being as one of the dominate contaminating fungi .The grow of *Khuskia oryzae* will attribute the formation of biofilm which has been verified to be a huge damage to airplane().The traditional methods for detection contaminating fungi (including *Khuskia oryzae*) were based on cultivation which required professional staff and aseptic laboratory. PCR methods compared to traditional methods has the advantage of repaid and no cultivation-based. However, PCR methods has its own limitation for requiring special and expensive instruments(Raikos et al 2011 and Silva et al 2008).

The oil tank and fuel system could perfectly meet the requirements of microbial growth and reproduction with water, suitable temperature and nutrients, therefor, the detection of dominate contaminating fungi were important for storage and fly safe. Here we developed a LAMP-LFD for detection of ITS sequence of *Khuskia*

oryzae has shown its superiority in sensitivity, specificity and convenient .The reaction condition of LAMP was at 63℃ for 60min. The sensitivity of LAMP-LFD for detection DNA of *Khuskia oryzae* was 230fg/μl which 10 time higher than LAMP and PCR. Compared to 5 other fungi control, only *Khuskia oryzae* could be accurate detected by LAMP and LAMP-LFD.In contrast, only two primers applied in the PCR reaction result in the poor specificity. he whole detection process of LAMP-LFD was 1.5h compared to PCR for 2.5h. In addition, AMP-LFD did not rely on the expensive instruments, therefor, t could reduce testing costs.

In conclusion, this LAMP-LFD assay developed in this study shown to be a rapid, accurate, and easy-to-perform method for detection of *Khuskia oryzae* in jet fuel. ur ultimate goal is like to conduct LAMP -LFD assay to be a new standard method for detection of dominate contaminating fungi of jet fuel .And the LAMP-LFD assay successfully applied in detection of *Khuskia oryzae* makes big step forward to this goal.

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