Rapid identification of four *Fusarium* spp. complex by high-resolution melting curve analysis and their antifungal susceptibility profiles

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Research Article

Keywords: *Fusarium*, fusariosis, high-resolution melting curve, identification, antifungal susceptibility

Posted Date: January 27th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1197822/v1>

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Abstract

Fusarium species are globally distributed filamentous ascomycete fungi that are frequently reported as plant pathogens and opportunistic human pathogens, leading to yield loss of crops, mycotoxin contamination of food and feed products as well as damage to human and livestock. Human infections of *Fusarium spp.* are difficult to treat due to broad antifungal resistance by members of this genus. Their role as disease-causing agents in crops and humans suggests a need for antifungal resistance profiles as well as a simple, rapid, and cost effective identification method. *Fusarium* strains were isolated from food and clinical samples. High-resolution melting curve (HRM) analysis was performed using specific primers targeting internal transcribed spacer (ITS) region, followed with evaluation of specificity and sensitivity. The antifungal susceptibility of four *Fusarium* species was studied using the Sensititre YeastOne method. HRM analysis revealed reproducible, unimodal melting profiles specific to each of the four *Fusarium* strains, while no amplification of the negative controls. The minimum detection limits were 100~120 copies based on a 2 µl volume of template. Clear susceptibility differences were observed against antifungal agents by different *Fusarium* isolates, with amphotericin B and voriconazole displayed strongest antifungal effects to all the tested strains. We developed a simple, rapid, and low-cost HRM-PCR method for identification of four *Fusarium spp.* (*F. oxysporum*, *F. lateritium*, *F. fujikuroi*, and *F. solani*). The antifungal susceptibility profiles supplied antifungal information of foodborne and clinical *Fusarium spp.* and provided guidance for clinical treatment of human infections.

Introduction

As widespread plant pathogens or conditional pathogens of humans, *Fusarium spp.* can be isolated from soil and plants worldwide[1–3]. *Fusarium spp.* typically infect plant vasculature, disrupting water and nutrient transport to tissues, while also producing toxic secondary metabolites that add to their virulence, cumulatively leading to wilting, rot of various plant organs, and eventually death. In particular, *Fusarium oxysporum* causes foliar blight, a vascular disease that can kill plants and result in major production losses [4–6]. *Fusarium solani* is a causative pathogen of root rot disease in a variety of crops, and fruit rot in a subset of hosts [7]. *Fusarium fujikuroi* is a globally distributed pathogen that causes seedborne diseases of rice, such as bakanae disease, through fungal gibberellin production that results in aberrant stem elongation of the host [8]. By contrast, *F. lateritium* is pathogen of woody plants and trees, and has been isolated from several hosts as a causative agent of wilt, tip or branch dieback, and cankers [9].

In addition to their pathogenic effects in plants, *Fusarium* produce mycotoxins such as zearalenone, trichothecenes, fumonisin, and fusaric acid, which are dangerous to human and livestock health [7, 10]. Symptoms of poisoning can be detected 30-60 minutes after the ingestion of contaminated corn or wheat. *Fusarium* mycotoxins do not readily break down through digestion and can be difficult to detect, leading to diagnosis problems. Moreover, detoxification is a clinically complicated process, which makes the monitoring and early identification of fungal contaminants in food a priority for food safety and regulatory agencies [2].

However, approximately 70 *Fusarium spp.* are related to infections that cause fusariosis in humans [11, 12]. The rate of human fungal infections increases with the upward trend of immunocompromised population, leading to an increase in the morbidity and mortality rates due to fusariosis [13–17]. This could be due to the fact that most available antifungal drugs are ineffective against fusariosis [18]. Studies have revealed that flucytosine and echinocandins show poor inhibitory effects against *Fusarium* species [16, 19, 20]. Therefore, the antifungal susceptibility characteristics of this genus prone to cause human infections is crucial for the clinical treatment.

The traditional classification and identification method of *Fusarium spp.* mainly relies on its morphological characteristics, which is time-consuming and often takes 2 to 3 weeks to get final result. Molecular identification methods, such as sequencing, multiplex PCR, restriction fragment length polymorphism (RFLP) analysis, multiplex ligation-dependent probe amplification (MLPA), and pyrosequencing, were also used for species identification [17]. Although the above methods are widely used, it takes a long time and may delay the treatment, make the patient's condition worse, or fail to identify the pathogen in time and affect the timeliness of food safety monitoring. Besides, these methods cannot be performed at scale due to the particular requirements for clinicians.

Therefore, a fast, simple, and economical method for practical applications is desirable. High-resolution melting curve (HRM) analysis can be performed in a closed tube right after real-time quantitative PCR (qPCR). Different melting profiles result from various GC contents, DNA sequences, and lengths, facilitating the identification of small differences in the target sequences [21, 22]. HRM analysis has been used to successfully identify species from the following: *Trichinella* (8 species) [23], *Candida spp.* (23 species) [24], and *Aspergillus spp.* section Nigri isolates (5 species) [25].

In this study, we reported a qPCR-HRM method that uses a pair of primers to produce different melting profiles, allowing us to identify four important foodborne and clinical *Fusarium spp.* pathogens (*F. fujikuroi*, *F. lateritium*, *F. oxysporum*, and *F. solani*) based on differences in the melt profile of the ITS region of rDNA. Antifungal susceptibility of these *Fusarium* species were studied against 9 commonly used antifungal drugs.

Materials And Methods

Strain isolation and maintenance

Food samples were collected from different cities in China between 2018 and 2021. Foodborne fungi were obtained with a potato dextrose agar (PDA) medium (Land bridge, Beijing, China) and 0.05% chloramphenicol (Sangon Biotech, Shanghai, China) in laboratory conditions. The cultures were isolated into single colony after cultivation at 28°C for 3-4 days, followed by identification of ITS sequencing. The primer sequences were as follows:

ITS1 (forward): 5'-TCCGTAGGTGAACCTGCGG-3'

ITS4 (reverse): 5'-TCCTCCGCTTATTGATATGC-3'

Alignment of the ITS sequence was performed using National Center for Biotechnology Information (NCBI) nucleotide basic local alignment search tool (BLAST). The samples were preserved and maintained at the National Institute for Communicable Disease Control and Prevention (ICDC, Beijing, China) using 25% glycerol (v/v) at -80°C. The clinical *F. solani* strain was previously isolated and identified by our lab. The collection sites of strains were recorded as the locations where the food samples or clinical materials were collected.

DNA extraction

Fusarium spp. samples were all grown for 7 days on Sabouraud's agar (SDA) medium. The cultured cells were transferred into centrifuge tubes with zirconium dioxide beads ($\Phi 1$ mm and $\Phi 3$ mm) and lysis solution (20 mM Tris, 5 mM EDTA, 400 mM NaCl), after which the cells were shaken at low temperature with a biological sample preparation system (Life Real, Hangzhou, China). After cell disruption, tubes were incubated at 95°C for 15 min, followed by cooling at 4°C for 2 h. Supernatant containing crude DNA extraction was transferred into new 1.5 ml tubes after centrifugation at 4°C (12000 rpm, 10 min), which was subsequently used as amplification templates.

HRM primers and plasmids

The ITS region of fungal rDNA bear little pressure of natural selection in the process of evolution, showing a wide range of sequence polymorphisms, which makes ITS sequence suitable for the molecular identification of fungal species [26, 27]. Therefore, we assessed the ITS1-ITS2 region sequences from the four *Fusarium spp.* published in GenBank and designed several primer pairs using the Lasergene Seqman software.

Gradient PCR was first performed using the DNA templates previously extracted and agarose gel electrophoresis was used to screen two primers capable of amplifying all four species. The primers sequences are as follows:

forward primer: 5'-CATGCCTGTTGAGCGTCATT-3'

reverse primer: 5'-CGAGGTCAACATTGAGAAGT-3'

The alignment of the amplified fragments and primer positions is shown in Figure 1. Plasmids were synthesized by submitting the sequences of the amplified fragments to Sangon Biotech (Shanghai, China) Co., Ltd.

Establishment of the HRM method

qPCR was carried out in a 30 µL reaction mixture with 11.2 µl ddH₂O, 0.9 µl of each forward primer and reverse primer, 15 µl HRM analysis mix (TIANGEN, Beijing), and 2 µl DNA template. Conditions for PCR are shown as following: 95°C for 10 min, followed by 35 cycles of 95°C for 15 s, 58°C for 25 s, and 72°C for 25 s. Cooling and heating speeds were 1.6°C/s, while HRM ramping was conducted according to the following: 95°C 10s, 60 °C 1 min, 95°C 10 s, 60 °C 1 min, with cooling and heating speeds of 1.6°C/s. Fluorescence data were obtained at 0.025°C increments once a second to produce the melting curves.

Specificity and sensitivity of HRM method

DNA of commonly found non-*Fusarium* fungi (including *Alternaria spp.*, *Aspergillus spp.*, *Candida spp.*, and *Penicillium spp.*) and *Fusarium* strains were used to perform the qPCR-HRM reaction and identify the specificity of the method.

Concentrations of plasmids containing target sequences were identified using Qubit, while the following formula was used to convert the concentration to copies per microliter (copies/µl): $\text{copies}/\mu\text{l} = (6.02 \times 10^{23}) \times (\text{ng}/\mu\text{l} \times 10^{-9}) / (\text{DNA length} \times 660)$. Plasmids were then gradually diluted using ultrapure water ($5 \times 10^6 \sim 5 \times 10^1$ copies/µl), after which qPCR-HRM analysis was conducted to identify the minimum detection threshold of this method.

Antifungal susceptibility testing

Antifungal susceptibility testing was carried out using Sensititre YeastOne YO10 panel (ThermoFisher, USA) according to the manufacturer's instructions. In brief, *Fusarium* isolates were inoculated on PDA medium for 7 days for conidia formation. The conidia were collected with cotton swabs and suspended in 0.85% saline containing 1% polysorbate. Tubes were placed still on a flat surface for 3-5 minutes to allow heavy particles to sink to the bottom. Conidia suspended in the upper layer was transferred to new tubes followed by vortexing. Spectrophotometry was used to assess conidia suspension by assessing optical density at 530 nm, varying from 0.15 to 0.17. 100 µl inoculum was added to 11 ml RPMI-1640, which was used as final inoculum. 100 µl final inoculum was added to each well of the YO10 plates. These plates were then covered with an adhesive sealing film and incubated at 28°C for 48 h. Finally, the plates were read visually using a reading mirror at the end of 48 h.

Results

The specificity of the HRM method for four *Fusarium* spp. complex

HRM analysis of ITS amplicons from four *Fusarium* strains (Table 1) and negative controls revealed melting profiles specific to each of the four strains, while none of the negative controls had amplification

products (Figure 2A). By taking the first derivative of the fluorescence signal, we obtained four reproducible, unimodal melting peaks, with different corresponding melting temperatures (T_m) (Figure 2B). We then used the T_m range from three independent experiments to calculate mean T_m ± SD as a distinct identifier or profile for each isolate to account for inter-assay variability while maximizing sensitivity and reproducibility. The mean T_ms for *F. oxysporum*, *F. lateritium*, *F. fujikuroi*, and *F. solani* were 83.559 ± 0.094°C, 86.145 ± 0.038°C, 86.757 ± 0.044°C and 88.966 ± 0.024°C, respectively. The maximum variation in T_m of the four reference strains was 0.094°C in triplicate assays. Since the mean T_m values for the four strains differed by at least 0.612°C, we concluded that this method could successfully discriminate among the four *Fusarium* strains.

Table 1
Source information for the representative of four *Fusarium spp.* complex

Sample ID	Species	Collection date	Source area	Matrix	Storage organization
F2470	<i>F. solani</i>	2018-11-6	Hainan, China	Clinical materials	National Institute for Communicable Disease Control and Prevention
F6762	<i>F. fujikuroi</i>	2020-4-20	Yunnan, China	Sugarcane	National Institute for Communicable Disease Control and Prevention
F7003	<i>F. lateritium</i>	2020-8-23	Beijing, China	Peach	National Institute for Communicable Disease Control and Prevention
F7519	<i>F. oxysporum</i>	2021-3-27	Hebei, China	Potato	National Institute for Communicable Disease Control and Prevention

The sensitivity of the HRM method for the four *Fusarium spp.* complex

We next conducted HRM analysis using serial dilutions of the ITS1-ITS2 region amplicons from each respective isolate to determine the sensitivity of this method. The results indicated that amplification curves with a characteristic "S" shape were generated within a copy number range of ~5 × 10¹-5 × 10⁶ copies/μl (Figure 3). Standard curves were generated using the Ct values plotted against template concentration, which indicated that the maximum Ct value in the linear range corresponded to a concentration of 5.24~6.33 × 10¹ copies/μl (6.33 × 10¹ copies/μl for *F. oxysporum*, 5.24 × 10¹ copies/μl for *F. lateritium*, 6.29 × 10¹ copies/μl for *F. fujikuroi*, and 5.27 × 10¹ copies/μl for *F. solani*). These results suggested that the lower limit of detection for this method was 100~120 copies based on a 2 μl volume of template.

The antifungal susceptibility of the four *Fusarium spp.* complex

To establish antifungal susceptibility profiles for these *Fusarium* spp. complex members, we tested the resistance of 26 isolates to a panel of antifungal compounds using Sensititre YeastOne YO10 plates. These assays established MIC values for each antifungal agent against the respective isolates, since no breakpoints in antifungal resistance have yet been reported among *Fusarium* species (Table 2). We observed that *F. solani* exhibited the strongest drug resistance, with only voriconazole and amphotericin B showing inhibitory effects on growth of the 13 *F. solani* isolates at the tested concentrations. Similarly, *F. oxysporum* and *F. fujikuroi* were resistant to all of the agents except posaconazole, voriconazole, and amphotericin B, while *F. lateritium* exhibited sensitivity to all tested drugs. These findings revealed clear differences in susceptibility to antifungal agents by different *Fusarium* spp. isolates.

Table 2
Minimum inhibitory concentration of *Fusarium* isolates in Sensititre YeastOne assays.

Species	No.	MIC range (µg/ml)								
		AND	MF	CAS	FC	PZ	VOR	IZ	FZ	AB
<i>F. oxysporum</i>	7	\	\	\	\	0.5-8	2-4	\	\	0.25-1
<i>F. solani</i>	13	\	\	\	\	\	2-8	\	\	0.5-4
<i>F. lateritium</i>	3	8	8	8	64	0.5-1	1-2	16	256	0.12
<i>F. fujikuroi</i>	3	\	\	\	\	2	2-4	\	\	2-4
MF, micafungin; CAS, caspofungin; FZ,5- flucytosine; PZ, posaconazole; VOR, voriconazole; IZ, itraconazole; FZ, fluconazole; AND, anidulafungin; AB, amphotericin B; \, No obvious inhibitory effect up to the highest concentration in the plate (See supplementary materials for detailed concentrations of each antifungal drug).										

Discussion

Fusarium species are globally distributed filamentous ascomycete fungi that are frequently reported as plant pathogens and opportunistic human pathogens[13, 14]. Species within *Fusarium* are causative agents of disease in a broad range of wild and cultivated plants. Fusaria are also known to produce mycotoxin food contaminants, such as trichothecenes and fumonisins, potentially resulting in severe economic losses for food producers [7]. By contrast, *Fusarium* spp. are occasionally reported to infect animal hosts as an opportunistic pathogen, resulting in fusarioses, which may be difficult to treat due to broad antifungal resistance by members of this genus [1, 12]. In addition, fusarioses is most commonly associated with corneal infections in humans [16]. Thus, their role as disease-causing agents in crops, and to a lesser extent in humans, suggests a need for antifungal resistance profiles as well as a simple, rapid, and cost-effective identification method.

Historically, distinguishing among *Fusarium* species has proven challenging based only on morphological characterization of isolates, biochemical tests, and disease symptoms [18]. Conventional

mycological practices for isolate identification, including morphological characterizations, biochemical assays, and genotyping by high throughput sequencing, multiplex PCR, or RFLP analysis, are time-consuming and labor-intensive, and are therefore unsuitable at large scale in clinical laboratories [17]. The HRM method established here relies on monitoring the decrease in dsDNA-binding fluorescent dye signal to find differences in denaturation rate of specific amplified DNA fragments. These denaturation rates indicate binding affinities that are affected by genetic variation at the single nucleotide level [21–25]. Unlike other PCR-based techniques, HRM does not require post-processing steps, such as gel electrophoresis or restriction digests, thus saving time and labor in genotyping. Furthermore, this method can be used to quantify DNA copy numbers in the host sample, and even at very limited initial template concentrations, suggesting its potential for clinical application.

Successful isolate discrimination by HRM depends on design of primers with sufficient specificity and length that the amplicons generate discernibly different melting profiles. Based on these principles, we designed primers for the ITS1-ITS2 region that should amplify 202 bp fragments. Our results showed that the first derivative peaks of melting curves for the four *Fusarium spp.* strains were both distinguishable by average melting temperature and unimodal. Sequence alignments of the *Fusarium spp.* complex (Figure 1) revealed numerous differences in the nucleotide sequences of the amplified fragments among strains, which resulted in the observed distinctions in T_m values and melting curves.

Fusariosis is increasingly recognized as a cause of morbidity or mortality worldwide [16]. In light of the continual growth of immunocompromised populations, this disease is likely to increase in prevalence due to increasing proportion of susceptible, high-risk patients [28]. Indeed, the incidence of *Fusarium* infections has increased in humans, as have incidence rates of other fungal infections. While the prevalence of *Fusarium* infections of the pulmonary system is lower than that attributable to *Aspergillus*, its patient outcomes are generally poorer, with higher rates of morbidity and mortality [15, 16]. This is considered to be resulted from the broad resistance of *Fusarium* species against commonly used antifungal agents [13, 20, 29]. Thus, *in vitro* evaluation of antifungal susceptibility of these strains provides significant guidance for appropriate antifungal drug selection in clinic.

Here, we conducted MIC testing for a panel of antifungals, such as amphotericin B, voriconazole, itraconazole, fluconazole, anidulafungin, and caspofungin, among which amphotericin B and voriconazole performed excellent antifungal effects with a mean MIC of 0.89 and 3 µg/ml for *F. oxysporum*, 1.5 and 4.61 µg/ml for *F. solani*, 0.12 and 1.67 µg/ml for *F. lateritium*, 2.67 and 3.33 µg/ml for *F. fujikuroi*, respectively. A survey examining invasive fusariosis conducted by the European Confederation of Medical Mycology found mean MIC values of 1.51, 7.60, 3.01, and 4.16 mg/L for amphotericin B, posaconazole, voriconazole, and itraconazole, respectively, against a set of 54 clinical isolates [30]. These findings are consistent with our results, which are in agreement with other studies showing that amphotericin B has lower MIC values *in vitro* than other antifungal compounds [20, 29].

In light of the fact that *in vitro* data do not necessarily correlate with *in vivo* effects, we emphasize that these MIC results still require validation with clinical data. Notably, despite its high *in vitro* MIC values,

reports suggest that voriconazole is effective in clinical interventions for fusariosis [30]. For instance, Nucci *et al.* described improved outcomes associated with the use of voriconazole among patients with invasive fusariosis [31]. However, a larger cohort study conducted over a longer observation period, with an expanded panel of isolates is necessary to improve our epidemiological understanding of this infection with robust statistical support. In addition, studies in stem cell lines and solid organ transplantation models are necessary to investigate the local epidemiology of invasive fusariosis.

Conclusion

In this study, we developed an HRM-PCR method for identification of four *Fusarium* spp. (*F. oxysporum*, *F. lateritium*, *F. fujikuroi*, and *F. solani*) that provides a relatively simple, fast and low-cost strategy for screening *Fusarium* isolates. Our results show clear differences in melting curves among these *Fusarium* species, with high specificity for amplification of the *Fusarium* spp. ITS1-ITS2 region. In addition, we performed antifungal susceptibility assays, thus providing guidance for the prevention and treatment of human infection with opportunistic *Fusarium* pathogens.

Declarations

Acknowledgements

This work was supported by National Key Research and Development Program of China (Grant Number 2018YFC1603801) and National Science and Technology Major Project of China (Grant Number 2018ZX10712001-006-007).

Conflicts of interest

The authors declare no conflict of interest.

Consent for Publication

All the authors agree to submit the manuscript for possible publication in “Mycopathologia”.

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Figures

Figure 1

Aligned sequences of the amplified ITS region of the four strains. Conserved bases are indicated by dark shading, variable sites are indicated by pink (one variation) and blue (two variations), and gaps within the sequence are indicated by hyphens. The binding sites of the primers are indicated by green rectangles.

Figure 2

A, The melting curves of the ITS1-ITS2 region amplicons of the four *Fusarium* spp. complex isolates. At the steepest point fluorescent signal decline phase, the four curves were distinct. B, First derivative peaks for each melting curve. The first derivatives of the melting curves were unimodal for all four species, with distinct mean T_m values of *F. oxysporum* (83.559°C), *F. lateritium* (86.145°C), *F. fujikuroi* (86.757°C), and *F. solani* (88.966°C), suggesting their use as unique identifiers for *Fusarium* spp. complex members. No amplification products were detected in the negative controls.

Figure 3

Analysis of HRM sensitivity. The amplification curves *F. oxysporum* (A), *F. lateritium* (B), *F. fujikuroi* (C), and *F. solani* (D). DNA templates were diluted to 10^6 , 10^5 , 10^4 , 10^3 , 10^2 , 10^1 copies/ μ l.

Figure 4

The standard curves for *F. oxysporum* (A), *F. lateritium* (B), *F. fujikuroi* (C), and *F. solani* (D). DNA templates were diluted to concentrations from 10^6 , 10^5 , 10^4 , 10^3 , 10^2 , 10^1 copies/ μ l. X axis: log value of DNA template concentration, Y axis: Ct value of DNA template concentration.

Supplementary Files

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