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Bead Mill Homogenization Streamlines Dermatomycoses Organism Identification by Drastically Reducing Sample Preparation Time

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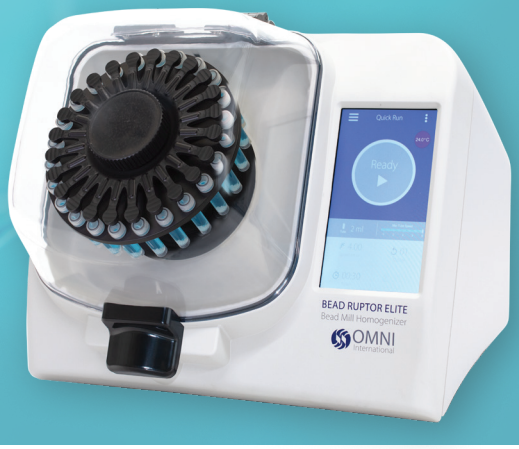
Bead Ruptor Elite™
Bead Mill Homogenizer



Summary

Pathogenic fungi, i.e. dermatophytes, are responsible for superficial infections known as dermatophytosis, which occurs in humans and animals (1, 2). These infections occur in nails, skin, and hair because of the high-affinity dermatophytes have for keratin and keratin-like structures (2-4). In addition to dermatophytes, non-dermatophyte molds and yeast can cause dermatomycosis. The pathogenesis of dermatophytosis involves secreting multiple enzymes, such as serine-subtilisins and metallo-endoproteases (fungalysins), formerly called keratinases. Enzymes such as hydrolases, lipases, and ceramidase are mucolytic enzymes that help penetrate keratin-rich tissue, providing nutrition to the fungi (2, 3). Human fungal infection is growing, creating a worldwide epidemic (4, 5). These fungi rarely cause infections in healthy individuals; however, their presence can be severe for individuals with depleted or compromised immune systems, such as those with HIV/AIDS, hence the importance of proper fungi identification and treatment (6, 7). Advances in laboratory technology offer time and cost savings; however, accurately identifying the presence of dermatophytes in humans and animals remains challenging.

Currently, the identification of dermatophytes and other pathogenic organisms is determined by mycological testing using culture, microscopic, and molecular techniques, which are time-consuming, not specific, or limited to the number of detectable fungi (9, 10). For example, one of the major complications associated with the macroscopic examination of fungi is the subjectivity of the morphological evaluation (4, 11). Likewise, microscopic examination of the structures found on fungi grown in culture may also be challenging to determine since morphology has been shown to change over time and may require laboratory personnel with extensive experience and expertise (11). In addition, culture has yielded negative results, even after visual examination confirmed the presence of arthrospores, suggesting possible isolation from non-dermatophyte fungi (Dermatomycosis) (4, 11). Molecular techniques such as traditional or real-time PCR have shown to be

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very selective, allowing accurate identification of fungi; however, the number of species that can be identified using the same primer set is limited and does not cover the full dermatophyte spectrum (4, 11). Therefore, species-specific identification is critical.

The number of dermatophytes identified continues to increase, partly due to advancing molecular techniques (12). The EUROArray Dermatomycosis system is a highly sensitive and specific molecular platform for identifying 50 dermatophyte species, 3 yeasts, and 3 molds. However, the time spent during sample preparation, specifically overnight digestion of nail samples, is the current lynchpin. Here we show a novel approach to sample preparation for identifying dermatophytes and non-dermatophyte origin. Our method included homogenization to disrupt the nail sample and any fungi present, producing a uniform homogenate. Nucleic acids of host and pathogenic origin are released, quantified, and identified as dermatophyte or non-dermatophyte using the EUROArray Dermatomycosis system for molecular genetic direct identification of the presence of dermatophytes, yeasts, and molds. Using homogenization, we significantly reduced the required sample preparation time thus improving sample throughput.

Materials and Methods

EQUIPMENT

OMNI

- Bead Ruptor Elite™ Bead Mill Homogenizer (Cat # 19-042E)
- Bead Ruptor Elite 2 mL Tube Carriage (Cat # 19-373)
- Bead Ruptor Elite Fingerplate (Cat # 19-370)
- 2 mL Hard Tissue Homogenizing Mix 2.8 mm Ceramic Beads (Cat # 19-628)

EUROIMMUN

- EUROArray Dermatomycosis Kit (Cat # MN 2850-2005)
- EUROArrayScanner System (Cat # YG 0601-0101)

HOMOGENIZATION SAMPLE PREPARATION

Dermatophyte-infected nails were purchased (Medical Diagnostic Laboratories LLC, NJ). Homogenization was performed using the OMNI Bead Ruptor Elite™ Bead Mill Homogenizer (OMNI, Cat # 19-042E). Nail samples (12 – 15 mg) were transferred to a 2 mL reinforced tube containing 2.8 mm ceramic beads (OMNI, Cat # 19-628), then 200 µL AL Buffer, 180 µL ATL buffer, and 20 µL Proteinase K were added. Tube contents were homogenized at 6.8 m/s for 4 x 30 second cycles with a one minute dwell. Tubes were centrifuged at 10,000 x g for 1 minute and incubated at 37 °C for 1 hour. Homogenization was repeated as mentioned above (6.8 m/s for 4 x 30 second cycles with a one minute dwell). Tubes were centrifuged at 10,000 x g for 1 minute. Samples were incubated in the heating block at 70 °C for 10 minutes. Sample tubes were removed from the heating block and pulse centrifuged two-three times to pull down the supernatant. Two hundred microliters of ethanol (95-100 %) was added to each sample, then vortexed for 15 seconds, followed again by pulse centrifuge two-three times to pull down the supernatant. Samples were transferred (600 µL) into the spin columns (Qiagen, Cat # 1113884). Samples were centrifuged in the collection tubes for 1 minute at 10,000 x g. Collection tubes were discarded and columns were placed into a new collection tube. AW1 buffer, 500 µL was pipetted into each column and centrifuged for one minute at 10,000 x g. Afterward, collection tubes were discarded and replaced with new collection tubes. AW2 buffer, 500 µL, was pipetted into each column, centrifuged for three minutes at 17,000 x g, collection tubes were discarded, and replaced with new collection tubes. Columns in collection tubes were centrifuged for one minute at 17,000 x g and discarded. A new reaction vessel, labeled, and column placed was inside it. AE Buffer, 50 µL, was pipetted into each column and allowed to sit for 5 minutes at room temperature. Columns were centrifuged in the reaction vessels for one minute at 10,000 x g. Columns were discarded, and reaction vessels were closed.

OVERNIGHT SAMPLE PREPARATION

Dermatophyte-infected nails were purchased (Medical Diagnostic Laboratories LLC, NJ). Sample preparation was performed using the QIAamp DNA Mini Kit (Qiagen, Cat # 700355). Nail samples (12 – 15 mg) were transferred to a snap cap tube (USA Scientific, Cat # 1615-5510), 80 µL of ATL Buffer added, followed by 20 µL Proteinase K to each sample. Samples were vortexed for 15 seconds, followed by centrifugation at 10,000 x g for 30 seconds (Labnet Mini-Centrifuge, Corning). Heating block was preheated to 56 °C. Samples were placed in the heating block and incubated overnight (13-16 hours). Afterward, heating block was set to 70 °C and 200 µL of AL Buffer was added to each sample, followed by vortexing samples for 15 seconds. Samples were incubated in the heating block at 70 °C for 10 minutes. Samples were removed from the heating block and pulse centrifuge two-three times to pull down the supernatant. Next, 200 µL of ethanol (95-100 %) was added to each sample, then vortexed for 15 seconds, followed again by pulse centrifuge two-three times to pull down the supernatant. Samples (600 µL) were transferred into the spin columns (Qiagen, Cat # 1113884). Samples were centrifuged in the collection tubes for one minute at 10,000 x g. Collection tubes were discarded and columns were placed into new collection tubes. AW1 Buffer, 500 µL, was pipetted into each column and centrifuged for one minute at 10,000 x g. Afterward, collection tubes were discarded and replaced with new collection tubes. AW2 buffer, 500 µL, was pipetted into each column, centrifuged for three minutes at 17,000 x g. Collection tubes were discarded and replaced with new collection tubes, columns were centrifuged in the collection tubes for one minute at 17,000 x g. Collection tubes were discarded. New reaction vessels were labeled, columns placed into the new reaction vessels. AE Buffer, 50 µL, was pipetted into each column, and columns were left to stand for 5 minutes at room temp. Columns were centrifuged in the reaction vessels for one minute at 10,000 x g. Columns were discarded and reaction vessel lids were closed.

PCR PREPARATION

PCR reagents A and B (EUROIMMUN, Cat # MM 2850-0106) from the EUROArray Dermatomycosis kit (EUROIMMUN, Cat # 2850-2005) were removed from the -20 °C freezer and placed in a white cooling rack stored at 4 °C. PCR master mix (11 µL Reagent A + 11 µL Reagent B/reaction) was prepared, inverted to mix, (not vortexed), followed by brief centrifugation of the master mix. PCR strip tubes were placed into the purple cooling rack. PCR master mix, 20 µL, was added to each tube, then eluted DNA, 5 µL, from each nail sample was added to each PCR tube. The negative control consisted of 5 µL dEPC water. The PCR tubes were placed in the thermocycler according to protocol (EUROIMMUN, Cat # 2850-2005).

EUROArray DERMATOPHYTE IDENTIFICATION

Hybridization

Important: Change gloves after PCR Preparation to Hybridization Step. Upon thermocycler start, hybridization station was turned on and set 55 °C to warm up. TITERPLANEs were dried thoroughly using the air compressor. TITERPLANEs were placed on the hybridization station under the black lids to warm up. Twenty minutes before thermocycler run was completed, EUROArray slides were removed from 4 °C to warm up to room temperature. After 10 minutes, the EUROArray slides were removed from their sealed packaging and placed on the TITERPLANE face down to warm up to 55 °C. Hybridization Buffer B (EUROArray Dermatomycosis kit) was removed from 4 °C to warm up to room temperature. Once the PCR is finished, tubes were removed and placed in the PCR tube rack. Hybridization Buffer B, 65 µL, was pipetted into the first PCR tube and mixed by pipetting up and down 3 times. Each PCR/Hybridization mixture was pipetted onto the TITERPLANE in its own field (well) on each slide. Each slide was placed face-down onto the sample and TITERPLANE. Black lids were placed over the hybridization station and slides incubated for 1 hour at 55 °C.

WASH AND DRY STEPS

All wash buffers (EUROIMMUN, Cat # ZM 0123-0101) were prepared according to manufacturer's instructions. Wash buffer concentrate (WBC) 1a was added, followed by distilled water, and WBC 1b then brought to 1,000 mL final volume in a glass bottle. WBC 2a was added, followed by distilled water, and WBC 2b into a 500 mL glass bottle and brought to a final 500 mL volume with distilled water. WBC 3 was added into a 500 mL glass bottle and brought to a final 500 mL volume with distilled water.

Four wash dishes (cuvettes) were filled with wash buffers. Two dishes with WB1 were filled, one dish with WB2, and one dish with WB3. After the hybridization was completed, EUROArray slides were removed allowing any excess sample to run down the side of the slide without running into other sample fields. The following was

performed for each slide: gently immersed the slide in WB1 for 10-15 seconds (cuvette 1), then placed the EUROArray slide in the glass carriage located in cuvette 2 containing WB1. The previous steps were repeated for the additional slides until all slides were in the glass carriage in cuvette 2 with WB1. Slides were washed for 1 minute in cuvette 2 with WB1. The glass carrier was placed in WB2 (cuvette 3) for exactly 2 minutes. The glass carrier was transferred directly from WB2 (cuvette 3) into WB3 (cuvette 4). The slides were washed in WB3 for a minimum of 5 seconds. Each slide was dried separately while the other slides remained in WB3. A slide was removed from WB3, dried with the small air compressor, and protected from light for 5 minutes at room temperature before scanning with the EUROArrayScanner.

Results

Sample preparation of the nails using homogenization was faster than the traditional overnight digestion protocol. In addition, molecular testing for dermatophytes, yeasts, and molds on the homogenized nail samples was performed using the EUROArray Dermatomycosis system. It was found to be 96 % accurate (23 out of 24 detected), showing that bead mill homogenization does not destroy nor augment molecular targets of detectability (Table 1).

The identification of *Fusarium solani* in 1 of the 3 samples using the overnight method can be misleading given that this species was not detected in any other sample from patient 8 in both methods, therefore reducing the chances of sample handling errors during the detection workflow. Further, *Fusarium* species are primarily found in soil and are also known to easily contaminate dermatomycosis samples during the handling process (13).

Sample ID	Replicate	Overnight Method		Bead Mill Homogenization Method		Agreement between methods
		Dermatophyte	Yeast/Mould	Dermatophyte	Yeast/Mould	
1	Replicate #1	N.D	*Candida parapsilosis*	N.D	*Candida parapsilosis*	3/3
	Replicate #2	N.D	*Candida parapsilosis*	N.D	*Candida parapsilosis*	
	Replicate #3	N.D	*Candida parapsilosis*	N.D	*Candida parapsilosis*	
2	Replicate #1	N.D	*Candida parapsilosis*	N.D	*Candida parapsilosis*	3/3
	Replicate #2	N.D	*Candida parapsilosis*	N.D	*Candida parapsilosis*	
	Replicate #3	N.D	*Candida parapsilosis*	N.D	*Candida parapsilosis*	
3	Replicate #1	*Trichophyton rubrum*	N.D	*Trichophyton rubrum*	N.D	3/3
	Replicate #2	*Trichophyton rubrum*	N.D	*Trichophyton rubrum*	N.D	
	Replicate #3	*Trichophyton rubrum*	N.D	*Trichophyton rubrum*	N.D	
4	Replicate #1	*Trichophyton rubrum*	N.D	*Trichophyton rubrum*	N.D	3/3
	Replicate #2	*Trichophyton rubrum*	N.D	*Trichophyton rubrum*	N.D	
	Replicate #3	*Trichophyton rubrum*	N.D	*Trichophyton rubrum*	N.D	
5	Replicate #1	N.D	N.D	N.D	N.D	3/3
	Replicate #2	N.D	N.D	N.D	N.D	
	Replicate #3	N.D	N.D	N.D	N.D	
6	Replicate #1	Trichophyton rubrum	N.D	*Trichophyton rubrum*	N.D	3/3
	Replicate #2	Trichophyton rubrum	N.D	*Trichophyton rubrum*	N.D	
	Replicate #3	Trichophyton rubrum	N.D	*Trichophyton rubrum*	N.D	
7	Replicate #1	*Trichophyton interdigitale, Trichophyton interdigitale/mentagrophytes, Trichophyton rubrum*	*Fusarium oxysporum*	*Trichophyton interdigitale, Trichophyton interdigitale/mentagrophytes, Trichophyton rubrum*	*Fusarium oxysporum*	3/3
	Replicate #2	*Trichophyton interdigitale, Trichophyton interdigitale/mentagrophytes, Trichophyton rubrum*	*Fusarium oxysporum*	*Trichophyton interdigitale, Trichophyton interdigitale/mentagrophytes, Trichophyton rubrum*	*Fusarium oxysporum*	
	Replicate #3	*Trichophyton interdigitale, Trichophyton interdigitale/mentagrophytes, Trichophyton rubrum*	*Fusarium oxysporum*	*Trichophyton interdigitale, Trichophyton interdigitale/mentagrophytes, Trichophyton rubrum*	*Fusarium oxysporum*	
8	Replicate #1	*Trichophyton interdigitale*	*Fusarium solani*	*Trichophyton interdigitale*	N.D	1/3
	Replicate #2	*Trichophyton interdigitale*	N.D	*Trichophyton interdigitale*	N.D	
	Replicate #3	*Trichophyton interdigitale*	N.D	*Trichophyton interdigitale*	N.D	
A-(-) Ctrl	Negative Control	Valid	Valid	Valid	Valid	1/1

Table 1. Identification results from overnight and bead mill homogenized samples. Agreement between results from both methods was 96 %, meaning that 23 out of 24 samples showed identical species detected on the EUROArrayScan. n=8 samples performed in triplicate (N.D – Not Detected).

Conclusions

There is a growing need to develop a plan for a workflow to identify dermatomycosis rapidly and reliably. Homogenization drastically reduced both sample preparation time, and the overall turn around time (TAT) from sample to result. Using homogenization, sample preparation was reduced to 80 minutes, compared with the overnight method of 13 hours. Homogenization reduced TAT to approximately 6 hours compared to the overnight method of approximately 18 hours. The substantial time savings benefit of homogenization for fungal detection was easy to implement.

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