

Full Length Research Paper

Identification of fungi from children's shoes and application of a novel antimicrobial agent on shoe insole

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13 strains of fungi were isolated from 12 shoes worn by children aged 6 to 12 for a period of more than half a year consisting of 5 strains of yeasts and 8 strains of molds. Through morphological observation and 18SrDNA sequence analysis, the 5 strains of yeasts were identified as follows: *Cryptococcus neoformans*, *Candida albicans*, *Cryptococcus albidus*, *Rhodotorula mucilaginosa* and *Candida utilis*. While the 8 strains of molds characterized and classified by point cropping, insert method and imprint method were respectively *Mucor* (2 subspecies), *Chain cell mold*, *Aspergillus* (3 subspecies) and *Penicillium* (2 subspecies). In order to protect children's feet from being infected by the fungi, a novel nano antimicrobial composite containing nano ZnO and isothiazolinone was prepared and was used to treat the children's insole by spraying. The inhibitory effects of the nano antimicrobial composite treated insole against yeasts and molds from children's shoes were evaluated by the inhibition zone and the inhibition ratio method. The results show that the insole treated with 2% nano antimicrobial composite could inhibit the growth of tested microorganism effectively, which could also achieve more than 97% inhibition ratio against the entire tested fungi.

Key words: Children's shoes, identification, fungi, nano antimicrobial composite.

INTRODUCTION

Shoes are vulnerable to be contaminated by micro-organisms in wearing and a more appropriate circumstance is provided by physical contact as well as sweat dipping. Hence various microorganisms can grow and breed in micro-environment of inner shoes. Medical statistics show that about 70% of adults have different degrees of beriberi in damp areas of China, especially young students and militaries (Mayan et al., 1999). Since children are usually engaged in many physical activities, they could secrete more sweat than adult for their vigorous metabolism (Dorman and Deans, 2000). As a result, more microorganisms in children shoes would propagate with high speed and produce an undesired odor, leading to stinking foot, mosses foot and a series of other diseases directly affecting children's physical and

mental health ultimately (Anita and Chris, 2010; Ian, 2010). Recent research shows that people's feet diseases are mainly caused by microorganisms. In our former research, a total of 13 bacteria were isolated from children's shoes (Li et al., 2011). However, up to now, there are little fungi isolated in children's shoes reported. If some specific pathogenic fungi could be isolated from children's shoes, some effective antimicrobial agents for different species of fungi could be selected to protect children's feet from being infected by fungi. For this purpose, yeasts from children's shoes were isolated and identified through morphology and 18SrDNA sequence analysis in this study. Molds were classified by point cropping, insert method as well as imprint method and identified to category referred to in fungi identification manual.

To decrease the opportunity to suffer from skin disease in feet and to eliminate the undesired odor caused by the fungi in children's shoes, it is necessary to control the fungi growth in shoes (Pranab et al., 2009). To solve

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these problems, a clean environment must be built in shoes, where shoe insole with antimicrobial activity can play an important role. Usually, the common antimicrobial processing for leather is adding fungicides in the fat-liquoring in leather manufacture to inhibit the growth of molds during long time storage and transport (Orlita, 2004). Few documents reported the inhibition of fungi in shoes through adding antimicrobial agents in the shoe insole. In addition, common leather fungicides such as 2-(thiocyanomethylthio) benzothiazole (TCMTB) may have some toxicity problems, for example, skin irritation (Fernández et al., 2002; Gu et al., 2007). Therefore, a new antimicrobial compound, nano antimicrobial composite, was synthesized in our laboratory, which had been used in shoe insole and showed long-term antimicrobial effectiveness in practical use for the period of six months. In this study, the application properties of the compound in shoe insole were investigated and the inhibitory effects of the treated leather against molds and yeasts were also evaluated by the inhibition zone and the inhibition ratio method (Gu et al., 2007).

MATERIALS AND METHODS

Materials

12 pairs of shoes worn by children aged 6 to 12 for more than half a year was selected as shoe samples. Potato medium agar (PDA) was used for the isolation of fungi (Guthrie, 2002). Luria-Bertani medium was used for 18SrDNA sequence analysis (Guthrie, 2002). The nano antimicrobial composite was synthesized by our laboratory and consisted of the following compounds: nano ZnO 0.8 g/L, isothiazolinetone 10 g/L, Tween 0.05 g/L, acrylic resin 3 g/L, and an initial pH of 6.0. The nano antimicrobial composite was stabilized by adding acrylic resin. The shoe insole used in this study was produced by a conventional process in factory. The tested fungi including yeasts and molds were isolated from children's shoes.

Isolation of fungi

The leather insole, plastic insole and leather lining from the shoes were cut into pieces, and then the pieces were put in a sterile conical flask with glass beads under sterile conditions. After the pieces were soaked in sterile water for 1 h, the conical flask was shaken in a vibrator (CHZ-82, produced by Jintan Fuhua Instrument Co., China) at 200 rpm for 3 h to disperse the cells of germs. When the cells were dispersed to single, 1 ml sample of cell suspension and 9 ml sterile water were added into a test tube together and mixed fully (Li et al., 2011). Then 1 ml mixture suspension was taken into 9 ml sterile water to obtain the 10^{-2} dilution. According to the same procedure, different concentrations of fungi 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} and 10^{-7} were obtained (Papamanoli et al., 2003). For isolating yeasts, 0.04 mg/ml ampicillin was added into sterile potato medium to inhibit the growth of bacteria and actinomyces (Papamanoli et al., 2003). When the ampicillin and medium were mixed uniformly, 15 ml of the mixture was poured into culture plates. After the medium was solidified by cooling, 0.2 ml diluents of 10^{-4} , 10^{-5} and 10^{-6} for each concentration were drawn into plates by a sterile pipette. Each concentration of germ suspension was inoculated for 3 plates and then plates were coated by a sterile glass rod uniformly. Lastly the culture plates were stored in a biochemical incubator (SPX-80BS-II,

Shanghai Medical Equipment Manufacture Co., China) under 28°C for 2 days (Li et al., 2011).

For isolating molds, procedures were the same to the method of isolating yeasts, but the difference was that 0.2 ml diluents of 10^{-2} , 10^{-3} and 10^{-4} for each concentration were drawn into plates by a sterile pipette.

Purification of the isolates

Colonies of yeasts and molds were observed with optical microscope (XZE-H, Chongqing Optical Instrument Co., China). According to different morphology of colonies, the single colony was picked and inoculated to the medium slant and in total there were 5 strains of yeasts and 8 strains of molds identified preliminarily. Then purified colonies were obtained by repeated streaking of the single colony on fresh agar plates and their morphology was recorded as a basis for classification in detail.

Morphological identification

Identification of yeast

The shapes, color of colonies and Methylene blue-chip were observed with optical microscope (Barnett et al., 1983).

Identification of mold

These were done by the following methods:

Point planting: a little of spores from the slant with an inoculation needle was picked and pointed on the appropriate location of a plate. Then the plate was inverted cultured in an incubator under 28°C for 4, 7, 10 days. When the mycelium grew well on the plate, it was stained with lactophenol cotton blue dye and observed with conventional microscopy (Breed et al., 1994).

Insert method: molds were inoculated to the plate and cultured with plugging sterile cover slip so that hyphae could grow attaching to the cover slip. After the growth of hyphae, it was set on a slide to observe by removing the cover slip gently (Breed et al., 1994).

Imprint method: colonies were printed on a cover slip and stained with lactophenol cotton blue dye, then observed with optical microscope directly (Breed et al., 1994). According to distinctly different morphology of colonies, molds could be identified to category referred to in the fungi identification manual.

Extraction of genomic DNA, amplification and analysis of 18SrDNA

According to the manufacturer's instructions, the extraction of total genomic DNA was performed using DNA extraction kit (Promega, USA). Polymerase chain reaction (PCR) amplification was also performed. A portion of the yeast 18SrDNA gene was amplified using the forward primers P1: 5'-GGAAGTAAAAGTCGTAACAAGG-3' and reverse primers P2: 5'-TCCTCCGCTTATTGATATGC - 3'. The reaction mixture was set up on ice as follows: 10 × Taq Buffer (with Mg^{2+}) 5 μL, dNTP 3.5 μL, forward primer and reverse primer each 1.5 μL, Taq DNA polymerase 0.5 μL, template DNA 2 μL, and adding ddH₂O up to 36 μL final volume. The PCR program was: 4 min at 94°C, 30 cycles were performed consisting of 45 s at 94°C, 45 s annealing and 30 s extension at 72°C, with a final extension step at 72°C for 10 min (Yang et al., 2011). Subsequently, the PCR product was separated by 0.8% agar-gel electrophoresis,

Table 1. Morphological characteristics of yeast.

Number	Shape	Color	Pattern	Reproduction
1#	Spherical	Initial white, later yellow	Irregular	Budding
2#	Oval	White	Regular	Budding
3#	Oval	Ivory	Irregular	Anterior
4#	Round	Pink	Irregular	Budding
5#	Round	Ivory	Regular	Budding

Table 2. Morphological characteristics of mold.

Number	Colonies' color	Individual pattern	Reproduction	Identification
6#	Initial white, later gray	Mycelium colorless, no diaphragm	Sporangiospore	<i>Mucor</i>
7#	Pink	Diaphragm present	Conidium	<i>Chain cell mold</i>
8#	Initial white, spore was black	No diaphragm	Sporangiospore	<i>Mucor</i>
9#	Initial white, later black flocking	Diaphragm present	Conidium, spherical	<i>Aspergillus niger</i>
10#	Yellowish	Diaphragm present	Conidium	<i>Aspergillus</i>
11#	Blue-green, flocking	Diaphragm present	Conidium, oval	<i>Penicillium</i>
12#	Green, flocking	Diaphragm present	Conidium, chain	<i>Penicillium</i>
13#	Initial white, later yellowish; flocking	Diaphragm present	Conidium, spherical	<i>Aspergillus Candidus</i>

and the band of expected size was cut off and purified with purification kit (Promega, USA). The expected bands were sequenced by BGI (Beijing, China). Furthermore, sequence identification was estimated by using the BLAST facility of the National Center for Biotechnology Information. All available subsets of 18SrDNA gene sequences were selected, analyzed and aligned with CLUSTALX 1.8 (Thompson et al., 1997).

Antimicrobial treatment of shoe insole

4 g nano ZnO, 50 g isothiazolinelcetone, 0.25 g Tween and 15 g acrylic resin were dissolved in a water solution, then the nano antimicrobial composite was stirred for 20 min in the high-shear dispersing emulsifier (T18 Basic, IKA Co., Germany). When the composite was stable after 2 h, the solution with the concentration of 2% of sterile shoe insole was evenly sprayed on the grain and flesh sides of the shoe insole with a common airbrush. After air drying, the treated leathers were cut into discs of 24.60 mm in diameter under sterile conditions for the test of inhibition zones. For the inhibition ratio test, the samples were cut into chips (5 × 5 mm).

Inhibition zone test

Using sterile culture medium, the tested solutions of molds and yeasts were prepared by diluting the mature fungi that have been isolated from children's shoes. For molds and yeasts, a sterile transfer loop was used to scrape one or two loops of pure mold spores or yeasts off the fresh cultures, and the mold spores or yeasts were well dispersed in 100 ml physiological saline solution (0.9%) after shaken for 2 h in a water-bath oscillator (CHZ-8, produced by Jintan Fuhua Instrument Company Limited). Then the tested solutions were prepared and the concentrations of molds and yeasts were 10^6 to 10^7 cfu/ml.

To prepare the plates containing yeasts or molds, 0.2 ml tested solutions was evenly coated by a spreader on the sterile plates. Then, using the sterile forceps, the tested discs of shoe insole was

placed in the center of the contaminated plates and lightly pressed by sterile cotton poles to make them tightly fixed to the plates. These samples were incubated in the incubator until clear inhibition zones appeared. The temperature in the incubator was 28°C for the molds and yeasts, and at the end of the incubation, the diameters of inhibition zones around discs were measured by a slide caliper.

Inhibition ratio test

In the inhibition ratio method, 2 g chips of the treated leather and 0.2 ml mixed seeded solution were added to a triangular flask containing 19.8 ml sterilized physiological saline solution. Then, the triangular flask was shaken for 2 h at 200 r/min in a water-bath oscillator. The colony forming units (cfu) of the remained solution before and after oscillation were determined by plating technique. The inhibition ratio (IR) of the treated shoe insole was then calculated by the formula;

$$IR = \frac{C_0 - C_t}{C_0} \times 100\%$$

Where, C_0 and C_t are the colony forming units of the solution before and after oscillation, respectively.

RESULTS AND DISCUSSION

Morphology identification

According to distinctly different morphology of colonies, there were 5 strains of yeasts labeled from 1# to 5#, respectively. The colony characteristics of the isolates could thus be helpful for their genetics identification (Table 1). More also, as can be seen from Table 2, there were many different categories of molds, the color of

Table 3. Identification of the sequences of 18SrDNA.

Number	Accession number	Species number	Similarity (%)
1#	235443	<i>Cryptococcus neoformans</i> var. Grubii H99	99.23
2#	559299	<i>Candida albicans</i> 1161	99.18
3#	100951	<i>Cryptococcus albidus</i>	99.22
4#	5537	<i>Rhodotorula mucilaginosa</i>	99.39
5#	4903	<i>Candida utilis</i>	99.50

Table 4. Diameters (mm) of inhibition zones against fungi.

Fungi	<i>Candida albicans</i>	<i>Rhodotorula mucilaginosa</i>	<i>Mucor</i>	<i>Aspergillus niger</i>
Control	0	0	0	0
Diameter (mm)	40.80	42.22	35.32	37.64

Which were significantly different from each other. According to Table 2, molds could be identified to category referred to fungi identification manual (Breed et al., 1994) and they were identified as follows: 6#, *Mucor*; 7#, *Chain cell mold*; 8#, *Mucor*; 9#, *Aspergillus niger*; 10#, *Aspergillus*; 11#, *Penicillium*; 12#, *Penicillium*; 13#, *Aspergillus candidus*.

Identification of the sequences of 18SrDNA

According to Table 3, the similarities of 5 strains 18SrDNA sequence all reach up to 99% compared with the standard strains in GenBank (Kolbert and Persing, 1999). Combined with the morphological characteristics, they could be identified as: 1#, *Cryptococcus neoformans* H99; 2#, *Candida albicans* 1161; 3#, *Cryptococcus albidus* C.E.Skinner; 4#, *Rhodotorula mucilaginosa* FC; 5#, *Candida utilis* Lodder and Kreger-van Rij. Published researches showed that *C. albicans* and *C. utilis*, which possess strong fermentation capacity, can decompose organic matter within sweat and cause undesired odor directly leading to skin and mucous membranes' disease such as thrush (Seneviratne et al., 2008; Weissman et al., 2000). Also, molds which are rich in starch and lipase enzymes could corrupt organic matter because of their saprophytic character. Molds can propagate and spread easily in the shoe's special micro-environment for they grow well under high humidity conditions (Mhetras et al., 2009). The results of identification are helpful for us to screen efficient antibacterial agents for producing antibacterial insole to protect children's feet effectively.

Inhibition zones

In the experiments, the antimicrobial agent was added to the shoe insole by spraying. The addition method of an antimicrobial agent is very important for its absorbability

and distribution in leather. Compared to the conventional addition method, the spraying in the experiments may resolve the problem of low absorptivity and uneven distribution.

The diameters of inhibition zones against fungi are listed in Table 4 and the pictures of inhibition zones against fungi are given in Figure 1. As shown in Table 4, the leather discs treated by nano composite antimicrobial agent can form clear inhibition zones. Furthermore, for different fungi, the diameters of inhibition zones of yeasts were larger than that of molds.

Inhibition ratio

Inhibition ratio is a quantitative method of evaluation. Compared to the results of inhibition zone method, the data of inhibition ratios (Table 5) showed that the shoe insole had better antimicrobial activities. After it was treated by nano composite antimicrobial agent, the insole treated with 2% nano composite antimicrobial could inhibit the growth of tested fungi effectively, which also could achieve more than 97% inhibition ratio against the entire tested fungi.

Conclusion

There were 13 strains of fungi isolated from 12 children's shoes, consisting of 5 strains of yeasts (*C. neoformans*, *C. albicans*, *C. albidus*, *R. mucilaginosa* and *C. utilis*) and 8 strains of molds (*Mucor*, *Chain cell mold*, *Aspergillus* and *Penicillium*), respectively. These results contribute to selecting efficient antimicrobial agents for children's insole. The antimicrobial compound containing nano ZnO and isothiazolinone was used to treat the children's insole by spraying and results indicated that the insole treated with 2% nano composite antimicrobial could show large inhibition zones and inhibit the growth of tested

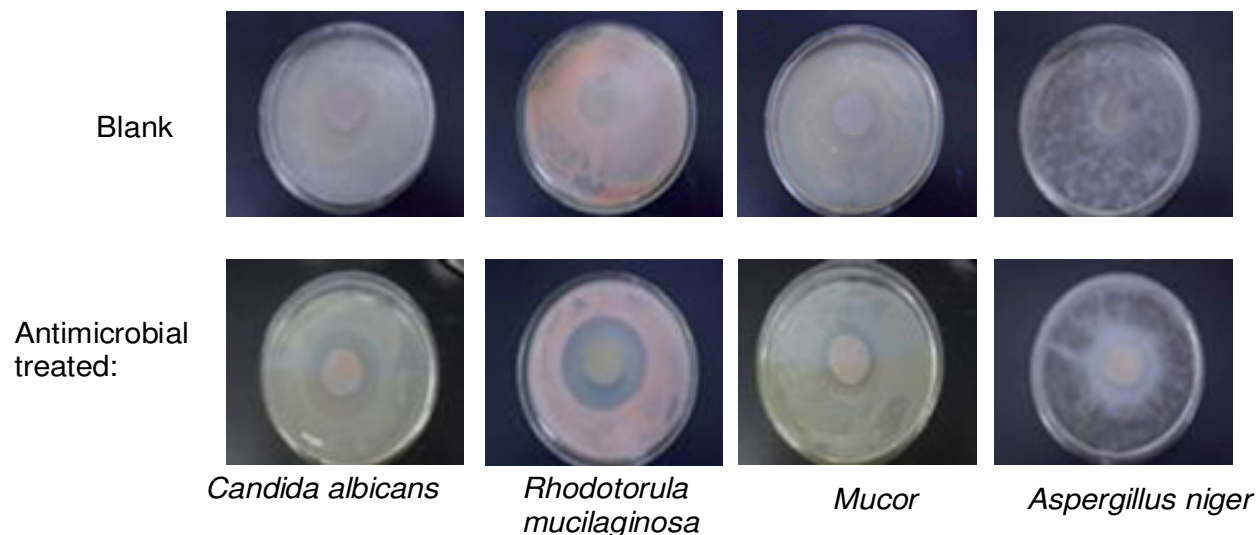


Figure 1. Pictures of inhibition zones against fungi.

Table 5. Inhibition ratio (%) of the shoe insole.

Fungi	<i>Candida albicans</i>	<i>Rhodotorula mucilaginosa</i>	<i>Mucor</i>	<i>Aspergillus niger</i>
Inhibition ratio	99.93	99.41	97.28	97.12

microorganism effectively by achieving more than 97% inhibition ratio against the entire tested fungi. Therefore, the new nano composite could be a potential antimicrobial agent applied to shoe insole to protect children's feet from being infected by microorganisms.

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