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Full Length Research Paper

Isolation, purification and identification of bacteria from the shoes worn by children

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13 strains of bacteria were isolated from 12 shoes that were worn by children aged 6 to 12 for more than half a year. Through morphological observation, physiological and biochemical measurements, as well as 16SrRNA sequence analysis, the bacteria were identified as follows: *Bacillus licheniformis*, *Bacillus subtilis* (5 subspecies), *Bacillus spore* (3 subspecies), *Bacillus anthracis*, *Staphylococcus aureus*, *Bacillus amyloliquefaciens* and *Bacillus thuringiensis*. The results may contribute to the selection of efficient antimicrobial agents for children's shoes and insole.

Key words: Children's shoes, bacteria, isolation, identification.

INTRODUCTION

Shoes are necessary for everyone, especially as they are worn by all in almost 2/3 of humans' life time. They are vulnerable to contamination by microorganisms when worn. Some published results show that a great amount of bacteria and fungi attach themselves to the feet and socks of humans, with the density of 5,000 to 100,000 colonies/cm^2, and more severely with a density of 10,000,000 to 1,000,000,000 colonies/cm^2 (Xu, 2006). It is these microorganisms that react with the sweat of humans and cause an undesired odor. However, various microorganisms could grow and breed largely in the micro-environment of inner shoes and would rightly cause incomparable beriberi by reacting with human's sweat. On the other hand, there was little information about these bacteria and fungi in shoes, although the pathogens which these microorganisms belong to have not been reported.

Since children are usually engaged in many physical activities, they could secrete more sweat than adults for their vigorous metabolism. As a result, more bacteria would breed and lead to more serious foot diseases (Cai, 2005). In the long-term result, a series of diseases could be induced, which would severely do harm to children's physical and mental health ultimately (Dorman et al., 2000). Shamez had successfully isolated a strain of *Staphylococcus* from children's shoes (Shamez and Mehdi, 2005), but up till now, no report has been made on the isolation of other bacteria from children's shoes. If the specific pathogenic microorganisms could be isolated and identified from the shoes, some effective anti-microbial agents for the different species of bacteria could be selected for materials used in making shoes to protect children's feet from being infected by the micro-organisms. For this purpose, the bacteria from children's shoes were isolated and identified with morphological observation, physiological and biochemical tests, extraction of genomic DNA, PCR amplification and analysis of 16SrRNA sequence.

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MATERIALS AND METHODS

12 pairs of shoes that were worn by children aged 6 to 12 for more than half a year, were selected as the shoes' sample. Beef extract peptone medium was used for the isolation of bacteria (Ausubel et al., 2005). LB medium, TE buffer (pH 8.0), CTAB/NaCl solution (pH 8.0) and dNTPs mixture were selected for 16SrRNA sequence analysis (Joseph et al., 2001).

Isolation of bacteria

The leather insole, plastic insole and leather lining from the 12 pairs of shoes were cut into pieces. Then, the pieces were put in a sterile conical flask with glass beads and 100 ml sterile water was added under sterile conditions. After the pieces were soaked in sterile

water for 1 h, the conical flask was shook in a vibrator (CHZ-82, Jintan Fuhua Instrument Co., China) at 200 rpm for 3 h to disperse the cells of germs. When the cells were singly dispersed, 1 ml sample of the cell suspension and 9 ml sterile water were added together into a test tube and mixed fully. 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 bacteria (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}) were obtained (Lu et al., 2006). For isolation of the aerobic bacteria, 0.05 mg/ml nystatin was added to sterile beef extract peptone medium to inhibit the growth of molds and yeasts (Papamanoli et al., 2003). When the nystatin 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^{-5}, 10^{-6} and 10^{-7} for each concentration were drawn into plates by a sterile pipette. Each concentration of germ suspension was inoculated for 3 plates, and then the 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) at 37°C for 2 days.

For isolation of the anaerobic bacteria, 15 ml of beef extract peptone medium 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 sealed by sterile Vaseline and placed in a vacuum dryer. After been vacuumed, the plates were stored in a biochemical incubator at 37°C for 2 days to enable the anaerobic bacteria grow largely on the plates.

Purification of the isolates

The morphology of colonies was observed with optical microscope (XZE-H, Chongqing Optical Instrument Co., China). According to different morphology of the colonies, the single colony of predominant bacteria was picked and inoculated to the medium slant, and in total, there were 13 strains of bacteria identified preliminarily. Then purified colonies were obtained by repeated streaking of the single colony on fresh agar plates and their morphology was recorded as the basis for classification in detail (Christine, 2002). In the experiment, there were no strict anaerobe bacteria on the vacuum dryer flat.

Morphological identification

After it was cultured at 37°C for 24 h, the strains were picked from the slants and were stained with gram as well as spore (Kandler et al., 1986). Then, the cell and spore of the strains were observed with optical microscope. At the same time, the common standard strains of *Escherichia coli* and *Bacillus* were selected as references (Kim et al., 2000).

Physiological and biochemical appraisal

There were a total of 10 trials based on the identification of a common bacterial system for appraising physiological and biochemical characteristics of the bacteria (Joseph et al., 2001). The common standard strains such as *E. coli* and *Bacillus* were also used as references.

Extraction of genomic DNA, amplification and analysis of 16SrRNA

According to the manufacturer's instructions, the extraction of the total genomic DNA was performed using DNA extraction kit (Promega, USA) and PCR amplification was performed. A portion of the bacterial 16SrRNA gene was amplified using the primers, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGCT ACCTTGTTACGACTT-3') (Mora et al., 1998). The reaction mixture was set up on ice and it included: 10 × Taq buffer (with Mg^{2+}) (5 µl), dNTP (3.5 µl), 1.5 µl each for forward and reverse primer, Taq DNA polymerase (0.5 µl), template DNA (2 µl) and an addition of ddH_2O up to 36 µl as the final volume. The PCR program was denaturized at 94°C for 5 min, and at 94°C for 30 s, while it was annealed at 57°C for 40 s and extended at 72°C for 90 s. These steps cycled a total of 32, while the program was finally extended at 72°C for 10 min (Mora et al., 1998). Subsequently, the PCR product was separated by 1% agar-gel electrophoresis, and the band of expected size was cut-off and purified with a purification kit (Promega, USA). The expected bands were sequenced by Huada Genomic Company (Beijing, China).

Sequence identification was initially estimated using the BLAST facility of the National Center for Biotechnology Information. All available subsets of 16SrRNA gene sequences were selected, analyzed and aligned with CLUSTALX 1.8 (Thompson et al., 1997).

RESULTS AND DISCUSSION

Morphology, physiological and biochemical identification

According to the different morphology of the colony (Figure 1), there were 13 strains of bacteria distinctly labeled from 1 to 13#, respectively. The morphological characteristics of the bacteria are shown in Table 1, and the physiological and biochemical characteristics of the bacteria are shown in Table 2. The colony characteristics of the isolates could thus be helpful for their genetics identification.

Identification of the sequences of 16SrRNA

According to Table 3, the similarities of the 13 strains of 16SrRNA sequence reached up to 98% when compared with the standard strains in the GenBank (Kolbert and Persing, 1999). Combining it with the morphological and physiological characteristics (Buchanan et al., 1984; Breed et al., 1994), these strains were identified as: *Bacillus licheniformis* CICC 10037 (1#); *Bacillus subtilis* 64-3 (2#); *Bacillus spore* CO64 (3#); *B. subtilis* SRS-15 (4#); *B. subtilis* W-9 (5#); *B. subtilis* I527 (6#); *B. subtilis* I3 (7#); *Bacillus anthracis* (8#); *Staphylococcus aureus* (9#); 1*Bacillus amyloliquefaciens* SDG-3 (10#); *B. spore* DC3158 (11#); *B. spore* LY (12#) and *Bacillus thuringiensis* 61436 (13#).

Among the strains isolated from the children's shoes, a strain of *S. aureus* accounted for 7.70%, while 12 strains of *Bacillus* accounted for 92.30%. Published researches show that most *Bacillus* can secrete amylase, lipase and protease to strongly decompose carbon pollutants, complex polysaccharides and proteins, and they also play an important role in decomposing water-soluble organic matter such as sweat, leading to foot odor (Wan, 2001;

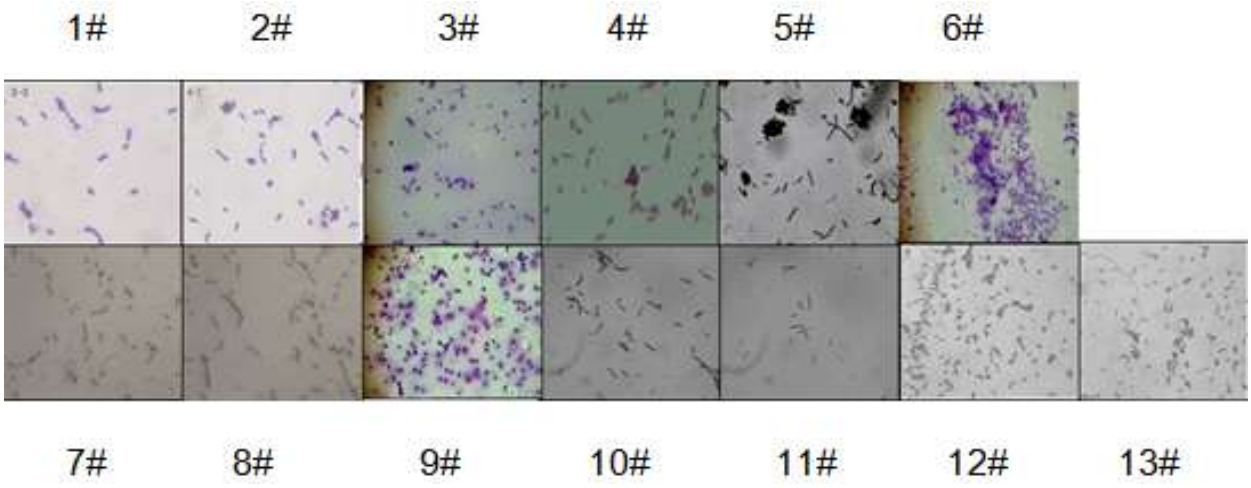

Figure 1. Morphology of the bacteria strains. The morphology of the 13 strains of the bacteria were observed with optical microscope (labeled from 1 to 13#), respectively; the size of bacteria observed with optical microscope were magnified by 1000 times.

Table 1. Morphological characteristics of bacteria.

S/N	Gram	Shape	Spore	Colonies color	Morphology	Transparency	Edge
1	G^+	Short rod	Had a spore	Yellowish	Round	Opaque	Irregular
2	G^+	Short rod	Had a spore	White	Irregular	Opaque	Irregular
3	G^+	Short rod	Had a spore	White	Round	Sub transparent	Regular
4	G^+	rod	Had a spore	Yellowish	Round	Opaque	Irregular
5	G^+	Long rod	Had a spore	White	Round	Opaque	Irregular
6	G^+	Short rod	Had a spore	Yellowish	Round	Opaque	Regular
7	G^+	Long rod	Had a spore	Yellowish	Round	Opaque	Regular
8	G^+	Long rod	Had a spore	Yellowish	Irregular	Opaque	Irregular
9	G^+	Spherical	No spore	Yellowish	Round	Opaque	Regular
10	G^+	Rod	Had a spore	Yellowish	Round	Opaque	Irregular
11	G^+	Rod	Had a spore	White	Round	Transparent	Regular
12	G^+	Rod	Had a spore	White	Round	Opaque	Irregular
13	G^+	Rod	Had a spore	Yellowish	Round	Opaque	Irregular

Bruce et al., 1988). *B. anthracis* could spread widely in dust pollution and easily cause skin anthrax (Quan et al., 2005). Also, *S, aureus* is the most common infection pathogen of the purulent, and it can produce toxin and cause serious harm, as aggressive bacteria, to the skin of people's feet (Proctor et al., 1995). In this study, 13 strains of bacteria from children's shoes were identified. The results of the identification will be helpful in screening efficient antibacterial agents for shoes and insole to protect children's feet effectively. Also, the results of the strain's identification will provide more useful information for the medical care of children's feet.

CONCLUSION

13 strains of bacteria were isolated from the children's shoes and were identified as *B. licheniformis*, *Bacillus subtilis* (5 subspecies), *B. spore* (3 subspecies), *B. amyloliquefaciens, S. aureus, Bacillus amyloliquefaciens* and *B. thuringiensis.* This is the first report on the bacteria isolated and identified from children's shoes in this area. Based on the results, some efficient anti-bacterial agents could be chosen against the pathogenic microorganisms for children's shoes and insole.

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Table 2. Characteristics of the physiology and biochemistry of bacteria.

S/N	1	2	3	4	5	6	7	8	9	10	11	12	13
Amylolysis	+	+	+	+	+	+	+	+	−	+	+	+	+
Gelatinolytic	+	+	−	+	+	+	+	+	+	+	+	−	+
Methyl red test	−	−	−	−	−	+	+	−	+	−	−	−	+
Indole test	−	−	−	−	−	−	−	+	−	−	−	−	+
Vp	+	+	−	+	+	+	+	+	+	−	−	−	+
Urea test	−	−	+	−	−	−	−	−	+	−	+	+	−
Sugar fermentation	+	+	+	+	+	+	+	+	+	+	+	+	+
Glucose fermentation	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannitol fermentation	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase test	+	+	+	+	+	+	+	+	+	+	+	+	+

+ Indicates positive reaction results; - indicates negative reaction results.

Table 3. Identification of the sequences of 16S Rrna.

S/N	Sequence number	Strain number	Species number	Similarity (%)
1	AY871102.1	CICC10037	*B. licheniformis*	99.29
2	EF472462.1	64-3	*B. subtilis*	99.14
3	DQ643066.1	CO64	*B. spore*	99.89
4	GU056808.1	SRS-15	*B. subtilis*	99.92
5	EU815066.1	W-9	*B. subtilis*	99.86
6	GQ199597.1	I527	*B. subtilis*	98.21
7	GQ199595.1	I3	*B. subtilis*	100
8	AY138383.1	2000031664	*B. anthracis*	99.47
9	AB114634.1	A9784	*S. aureus*	99.31
10	FG436406.1	SDG-31	*B. amyloliquefaciens*	99.98
11	GU121479.1	DC3158	*B. spore*	99.49
12	AY787805.1	LY	*B. spore*	98.54
13	FJ932761.1	61436	*B. thuringiensis*	99.48

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