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Full Length Research Paper

Ciprofloxacin and tetracycline susceptibility of lactobacilli isolated from indigenous children's feces

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To investigate the frequency of ciprofloxacin and tetracycline resistance lactobacilli in children feces, a total of 160 feces samples were cultured on Lactobacilli-selective Rogosa agar supplemented with 0.1 mg/ml of cycloheximide and 0.5% of $CaCO_3$, and identified *Lactobacillus* species were identified by analysis of the PCR sequenced-16S rRNA gene through BLAST against the deposited GenBank database. In these samples, 96 isolates were obtained and identified as belonging to 6 species, including *Lactobacilli plantarum, Lactobacilli helveticusi, Lactobacilli salivarius, Lactobacilli casei, Lactobacilli fermentum and Lactobacilli pentosus.* Strain-subtyping of these isolates by repetitive extragenic palindromic (REP)-PCR demonstrated a notable genotypic biodiversity of 65.6%. Antimicrobial susceptibility of ciprofloxacin and tetracycline had a wide different minimum inhibitory concentration (MIC) values in these isolates. The MIC50 and MIC90 of ciprofloxacin both was 64 µg/ml for both, while the MIC50 and MIC90 of tetracycline were 128 and 512 µg/ml. These results indicate that high-level resistant activity of ciprofloxacin and tetracycline among *Lactobacillus* species in indigenous children's intestines was prevalent in mountain district at the central area of Taiwan.

Key words: Indigenous child, ciprofloxacin and tetracycline resistance lactobacilli, Lactobacilli-selective rogosa agar, cycloheximide, antimicrobial susceptibility test, repetitive extragenic palindromic (REP)-PCR, minimum inhibitory concentration (MIC).

INTRODUCTION

Lactobacilli are members of the normal microflora existing in the gastrointestinal (GI) tract of human (Tannock et al., 1990). In general, the lactobacilli level in intestine was suggested to be an index of animals' healthy status. Nevertheless, the emergence of antibiotic resistance (AR) in this commensal bacteria (Mathur and Singh, 2005), after several decades' indiscriminative use of antibiotics in human medicine, has raised an important public health issue.

Ciprofloxacin is one of the most widely used of antibiotic against intestinal Gram-negative bacteria, enterococci and Bacteroid fragilis in clinical activity (Krueger et al., 1997; Sullivan et al., 2004). It is a second-generation fluoroquinolone class drug, synthetic broad-spectrum antibiotics and that inhibit DNA gyrase and

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topoisomerase activity. The action of ciprofloxacin on gastrointestinal infection is secreted through the mucosa to intestinal lumen that may affect indirectly the existence of lactobacilli amounted and influence the ecological balance of intestinal bacteria. Therefore, it is important to understand the resistant extent of ciprofloxacin in lactobacilli in human medicine.

Tetracycline is a broad spectrum antibiotic exhibiting activity against a wide range of Gram-positive and Gram-negative bacteria. This favorable antimicrobial property, along with the low cost and absence of major adverse side effects, let tetracycline be the most commonly used antimicrobial in clinical therapy in some countries (McDonald et al., 2001; Shlaes, 2006). Abuse of tetracycline has led to severe resistance. It is urgent for strict management of tetracycline use in human health maintenance and life extension.

MATERIALS AND METHODS

Isolation of *lactobacillus* spp.

A total of 160 feces samples of 8 to 12 year school-age children from 2 country's elementary schools located at the central area of Taiwan were randomly collected from May, 2008 to June, 2009. The stool specimens were collected with sterile container on ice and shipped to the laboratory within three hours. All samples were made and homogenized with 10 g of glass beads (4 mm diameter; Fischer scientific, Den Bosch, The Netherlands) in 9 ml of sterile PBS buffer on ice for 15 min. The homogenized suspension was filtered with sterile gauze to remove any large particles and debris, diluted ten times with sterile PBS buffer and 100 µl of dilutions were spread on Lactobacilli-selective Rogosa agar (LS-Rogosa agar, Difco, USA) supplemented with 0.1 mg/ml of cycloheximide (Sigma) and 0.5% of $CaCO_3$ (Sigma). After anaerobic incubation at 37℃ for 48 h, only one colony per specimen was randomly selected and cultivated in man rogosa sharp (MRS; Difco) broth. All isolates were tentatively identified as lactobacilli by Gram stain, oxidase, catalase and mobility tests and stored in 20% glycerol at −80℃.

Identification of *Lactobacillus* species by sequence analysis of 16S rRNA gene

Bacteria were grown overnight on MRS agar at 37℃ in an anaerobic atmosphere. Chromosomal DNA was extracted with a DNeasy Tissue Kit (Qiagen). A pair of *Lactobacillus* genus-specific PCR primers (Dubernet et al., 2002), R16-1 (5'-CTT GTA CAC ACC GCC CGT CA-3') and LbLMA1-rev (5'-CTC AAA ACT AAA CAA AGT TTC-3') corresponding to positions of 1400 to 1419 and 1597 to 1617 of the 16S rDNA, was used to amplify the specific fragment following conditions as described by Kwon et al. (2004). The products were then sequenced with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, CA and USA) and subjected to the sequence similarity analysis through BLAST (http://www.ncbi.nlm.nih.gov/blast) against the deposited GenBank database. Species identity of the isolates was determined on the basis of the highest scores (> 98%).

Strain typing

To distinguish strains of each species, total DNA was extracted from different isolates and typed by the repetitive extragenic palindromic (REP)-PCR using the (GTG)5 primer as described by Gevers et al. (2001). Gel images were exported into the pattern analysis software package Gelcompar II™ (version 5.00; Applied Maths BVBA, Sint-Martens-Latem, Belgium) for processing. Calculation of similarity of the PCR fingerprinting profiles was based on the band-based Dice correlation coefficient (1.0% optimization and 1.0% position tolerance). A dendrogram was deduced from the matrix of similarities by the unweighted pair group method using arithmetic average (UPGMA) clustering algorithm.

Minimum inhibitory concentration (MIC) for ciprofloxacin and tetracycline

The MIC for tetracycline was determined by standardized broth microdilution procedure in Iso-Sensitest broth (90%) and MRS broth (10%) (Klare et al., 2005). Ciprofloxacin was used at concentrations ranging from 1 to 64 µg/ml and tetracycline was used at concentrations ranging from 1 to 1024 µg/ml. Bacterial inocula were adjusted to a final concentration of 1 × 106 CFU/ml. Equalized volume(100 µl) of cell suspension and ciprofloxacin/tetracycline were inoculated into each well of a Nunclon Sterile plate. The MIC was defined as the lowest ciprofloxacin/tetracycline concentration producing no visible growth. For the control of reproducibility, four strains of *Lactobacillus*, including L. plantarum ATCC 14917, *L. helveticus* ATCC 15009, *L. pentosus* ATCC 8041, and *L. casei* ATCC 393, were used as reference against ciprofloxacin, four strains of *Lactobacillus*, including L. reuteri ATCC23272, *L. plantarum* ATCC 14917, *L. gasseri* ATCC 33323 and *L. intestinals* ATCC 49335, were used as reference against tetracycline in antimicrobial susceptibility test.

RESULTS

Isolation and identification of *Lactobacillus* species from indigenous child feces

A total of 160 samples analyzed were found to have typical white colonies surrounded with clear halos on the *Lactobacillus* selective medium. One colony per sample was then randomly picked and subjected to the morphological and biochemical analysis (Table 1).

Further species identification, employing PCR and sequence analysis of the 16S rRNA gene, a total of 159 strains were able to identify distinguish into 6 *Lactobacillus* species (Table 1), including *L. plantarum* (n = 72, 45.4%) was the most predouminate strain, *L. helveticusi* (n = 15, 9.4%), *L. salivarius* (n = 5, 3.1%), *L. casei* (n = 2, 1.3%), L. fermentum (n = 1, 0.6%) and *L. pentosus* (n = 1, 0.6%), sequentially.

Strain typing

REP-PCR analysis, using the (GTG) 5 primer, of the 159 isolates of lactobacilli demonstrated a notable genotypic heterogeneity of (GTG) 5 pattern (strain) among each species. As shown in Table 2, the percentages of (GTG) 5-strain biodiversity (BD) of the 6 species examined were found to range from 61.1 to 100%, with an average of 65.6%.

Table 1. Isolation and species identification of lactic acid bacteria from indigenous children feces at the central. area of Taiwan.

Procedure	Number of isolates (%)
LS- Rogosa Agar[a] + 0.5 mg/ml Cycloheximide + 0.5% $CaCO_3$ Human feces (n = 160[b])	
↓	
Gram stain (+), Catalase test (-), Motility test (-), Oxidase test (-)	
↓	
Species identification[c] (results)	159
L. plantarum	72 (45.4)
L. helveticusi	15 (9.4)
L. salivarius	5 (3.1)
L. casei	2 (1.3)
L. fermentum	1 (0.6)
L. pentosus	1 (0.6)
Others	63 (39.6)[d]

[a] Lactobacilli-selective Rogosa agar (Difco). [b] One colony per human was selected from the 160 feces samples. [c] Species identification was done by PCR assay using a pair of *Lactobacillus* genus-specific primers, followed by the sequence similarity analysis of partial 16S rRNA gene through BLAST (http://www.ncbi.nlm.nih.gov/blast) against the deposited GenBank database. Species identity of the isolates was determined on the basis of the highest scores (> 98%).[d] These strains were classified as others which were unable to be identified as belonging to the genus of *Lactobacillus* in this assay.

Table 2. Number of strains identified by (GTG)5 rep-PCR and the percentage of biodiversity (BD) among isolates of different species.

Species	Children feces specimen		
	No. isolates	(GTG)5 rep-PCR	
		Strains	BD (%)
L. plantarum	72	44	61.1
L. helveticus	15	11	73.3
L. salivarius	5	4	80
L. casei	2	2	100
L. fermentum	1	1	-[a]
L. pentosus	1	1	-[a]
Total	96	63	65.6

-[a]: meaningless to calculate the BD%.

Determination of the MIC

The MIC values for ciprofloxacin were summarized in Table 3. The MIC values ranged between 4 and 64 μg/ml in our strain higher than the breakpoint of *Lactobacillus* in Scientific Committee for Animal Nutrition (SCAN, 2003). The results indicated that a very high-level of antimicrobial activity against ciprofloxacin in our isolates.

The MIC values for tetracycline of the 63 strains were summarized in Table 4. The MIC values ranged between 16 and 1024 μg/ml. Both MIC50 (128 μg/ml) and MIC90 (512 μg/ml) values had been showed high-level resistant activity, which indicated an ecological distribution of high tetracycline resistant potency lactobacilli in indigenous child intestines.

DISCUSSION

In the subsequent species identification of the randomly selected colonies, 63 out of the 159 tentatively *Lactobacillus*-diagnosed isolates (39.6%, 63/159) were not recognized by the *Lactobacillus*-specific primers (Dubernet et al., 2002), which could identify a total of 23 *Lactobacillus* species in 2002. Given the fast growing species number of *Lactobacillus*, that is., > 70 species in 2010 (Narayan et al., 2010), and also the complex microbiota in child feces sample that usually contain a lot of unknown bacteria. To study these peculiar unidentifiable isolates may give a better insight into the lactobacilli in child intestines.

In this study, a total of 6 *Lactobacillus* species were

Table 3. MIC[a] data of ciprofloxacin for *Lactobacillus* species isolated from children feces samples.

Species	No. strains tested	(μg/ml)		
		MIC range	MIC_{50}	MIC_{90}
L. plantarum	44	32-64	64	64
L. helveticus	11	16-64	64	64
L. salivarius	4	32-64	32	64
L. casei	2	4-64	4	64
L. fermentum	1	64	64	64
L. pentosus	1	64	64	64
Total	63	4-64	64	64

[a] The MIC for ciprofloxacin was determined at concentrations ranging from 0.5 to 128 μg/ml by standardized broth microdilution procedure in Iso-Sensitest broth (90%) and MRS broth (10%) as described by Klare et al. (2005). For the control of reproducibility, four strains of *Lactobacillus*, including *L. plantarum* ATCC 14917, *L. helveticus* ATCC 15009, *L. pentosus* ATCC 8041, and *L. casei* ATCC 393, were used as reference in each batch of test. The MIC values for the reference strains were determined as > 64, 64, 32, and 16, respectively, in our study.

Table 4. MIC[a] data of tetracycline for *Lactobacillus* species isolated from children feces samples.

Species	No. strains tested	(ug/ml)		
		MIC range	MIC_{50}	MIC_{90}
L. plantarum	44	16-512	64	256
L. helveticus	11	32-1024	256	512
L. salivarius	4	512-1024	512	512
L. casei	2	256-1024	256	1024
L. fermentum	1	16	16	16
L. pentosus	1	256	256	256
Total	63	16-1024	128	512

[a] The MIC for tetracycline was determined at concentrations ranging from 1 to 1024 μg/ml by standardized broth microdilution procedure in Iso-Sensitest broth (90%) and MRS broth (10%) as described in Klare et al. (2005). For the control of reproducibility, four strains of *Lactobacillus*, including *L. reuteri* ATCC23272, *L. plantarum* ATCC 14917, *L. gasseri* ATCC 33323 and *L. intestinals* ATCC 49335, were used as reference in each batch of test. The MIC values for the reference strains were determined as 32, 64, 4 and 2, respectively, in our study.

identified, the most numerous species being recognized in a single survey. Similar finding were observed in some studies (Axelsson and Lindgren 1987; Angelis et al., 2007; Korhonen et al., 2007; Khunajakr, 2008; Guo et al., 2010). *Lactobacillus* plantarum was the most predominant specie (45.4%) (Table 1) in our study, it supported that it is one of the ubiquitous members of the naturally-occurring gut bacteria (Lähteinen et al., 2010). As for the other 5 species including *L. helveticusi, L. salivarius, L. casei, L. fermentum* and *L. pentosus* were described as dominant species in some studies concerning with human GI tracts (Pryde et al., 1999; Du Toit et al., 2001; Jassim, 2003; Yin and Zheng, 2005; Angelis et al., 2007; Khunajakr, 2008; Guo et al., 2010). Worthy of note was that *L. helveticus* was the first showed to be the second predominate (9.4%) inhabitants in human feces specimens. However, contrary to this new resident being identified, a well-known species in some human GI investigations that is; *Lactobacilli acidophilus*, was surprisingly not found in this survey.

Antimicrobial resistance of ciprofloxacin was a variable 17 to 95.3% from isolates of lactobacilli (Mändar et al., 2001; Pérez et al., 2005) except for *L. fermentum* and *Lactobacilli brevis* were 100% resistance. Their findings in the resistant activity were notably different from all strains unsusceptibliity in our study. Calculation from the available published data (Korhonen et al., 2007), a very low values of ciprofloxacin MIC range and MIC50 (that is, 0.125 to 16 and 2, respectively) (Nawaz et al., 2011). MIC range and MIC90 (that is, 2 to 32 and 32, respectively) (Malathum et al., 1999). In 2003 SCAN indicated 4 μg/ml is a breakpoint for ciprofloxacin in *Lactobacillus* species. Compared with the correspondent ciprofloxacin MIC range, MIC50 and MIC90 found in our study (Table 5), it showed higher resistance potency in our lactobacilli.

Nawaz et al. (2011) showed low values of tetracycline MIC90 (that is, 0.25 to > 128 μg/ml, respectively) in their numerous *Lactobacillus* species isolated from fermented food products in China (Korhonen et al., 2007).

Table 5. MIC of ciprofloxacin and tetracycline resistant lactobacilli among child fece specimens.

Antibiotics	Species	Number of strain(s)	MIC (ug/ml)										
			1	2	4	8	16	32	64	128	256	512	1024
Ciprofloxacin	*L. plantarum*	44						3	41				
	L. helveticus	11					1		10				
	L. salivarius	4						2	2				
	L. casei	2			1				1				
	L. fermentum	1							1				
	L. pentosus	1							1				
Tetracycline	*L. plantarum*	44					10	9	7	12	2	4	
	L. helveticus	11						1	4		1	4	1
	L. salivarius	4										3	1
	L. casei	2									1		1
	L. fermentum	1					1						
	L. pentosus	1									1		

Compared with the correspondent tetracycline MIC50 and MIC90 found in our study (Table 5), it showed the high resistance potency in our lactobacilli. These values indicated that drugs management need more close confinement in clinical used.

From our previous study Chang and Tsai (2011) found that pigs have higher resistance potency in ciprofloxacin and tetracycline. It may transfer from pig to human by food or environmental vectors. To prevent or reduce the drug resistance in animal is an urgent issue.

We suspected that the respective quinolone resistance-determining region (QRDR) mutation and a variety of tetracycline resistant genes were the major factors in these antibiotic resistant bacteria.

In conclusion, this study conferred the resistant activity of ciprofloxacin and tetracycline in indigenous child intestinal lactobacilli. These results indicated that high-level resistant activity of ciprofloxacin and tetracycline among *Lactobacillus* species in indigenous child intestines and antibiotics abuse was appeared to mountain district at the central area of Taiwan. Consequently, the enforcement of prudent use of antibiotics must be an important strategy in controlling the dissemination and emergence of antibiotic resistance.

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