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Gibel carp *Carassius auratus* gut microbiota after oral administration of trimethoprim/sulfamethoxazole

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ABSTRACT: Trimethoprim/sulfamethoxazole is widely used in the treatment of infectious diseases caused by bacterial pathogens in aquaculture. However, the practice of antibiotic administration can promote the emergence of resistant strains of bacteria and result in a wane in efficacy over time. The objective of this study was to assess the effect of oral treatment with trimethoprim/sulfamethoxazole on the gastrointestinal (GI) microbiota of healthy gibel carp and those affected with bacterial enteritis. By using denaturing gradient gel electrophoresis (DGGE), the changes in the predominant bacterial communities were directly depicted for the first time. The main findings were (1) *Actinobacteria*, *Firmicutes* and *Proteobacteria* were the predominant phyla in the healthy gibel carp intestine; (2) administration of antibiotics had a more profound impact on the intestinal microflora of healthy fish than of the diseased ones; and (3) *Enterobacteriaceae* might be one of the major drug-resistant bacteria in the gibel carp intestine. This study provides an insight into the effect of antibiotic treatment on the establishment and colonization of fish GI microbiota and speculates on some possible drug-resistant bacteria.

KEY WORDS: Gastrointestinal microbiota · Gibel carp · Trimethoprim/sulfamethoxazole · PCR–DGGE

INTRODUCTION

The fish gastrointestinal (GI) tract is a very complex and dynamic microbial ecosystem that is very important from nutritional, physiological and pathological points of view (Nayak 2010). Disturbances in this ecosystem can lead to a variety of conditions, such as colonization by potentially pathogenic bacteria (Vollaard & Clasener 1994). Within the rapidly growing aquaculture industry, bacterial enteritis has become one of the most serious diseases in China (Gao et al. 2009). This infection has rapidly spread and causes mass mortality due to hygienic problems arising from increased fish population density and lack of sanitary barriers between fish in the traditional aquaculture system (Naylor et al. 2000). The use of antibiotics remains totally unrestricted in China and other developing countries (Cabello 2006).

Trimethoprim/sulfamethoxazole is widely used for long-term prophylaxis and treatment of GI tract infections (Masters et al. 2003). However, the selective pressure exerted by antimicrobial use might result in a transfer of antimicrobial resistance genes within and across bacterial species and antimicrobial use is considered crucial to the emergence and dissemination of antibiotic-resistant strains in the intestinal tract (Donskey 2006). By streaking the pure culture on antibiotic plates, antibiotic-resistant bacteria have been identified in the GI tract and aquaculture

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settings (Clapper & Meade 1963, Hatha et al. 2005). Serial dilution of phenicol onto agar plates has also been used to investigate the resistance of fish pathogenic bacteria (Michel et al. 2003). However, these methods failed to reflect the total microbial community structure in the complex and dynamic ecosystem of the intestinal tract.

Molecular biology techniques based upon direct analysis of 16S rDNA can improve the characterization of these communities (Muyzer & Smalla 1998). For example, polymerase chain reaction–denaturing gradient gel electrophoresis (PCR–DGGE) of variable V3-region on 16S rDNA is a rapid and easy approach to evaluate microbial diversity and is capable of detecting the dominant bacterial species in environments (Muyzer et al. 1993). In this study, we used PCR–DGGE technology to monitor the gut microbiota community changes in healthy and diseased gibel carp *Carassius auratus* upon trimethoprim/sulfamethoxazole exposure. The shifts in bacterial populations and/or those bacteria unaffected by these antibiotics were also assessed.

MATERIALS AND METHODS

Sample collection and pathogen identification

About 60 healthy gibel carp of approximately the same size (800–1000 g) cultured in one large cage (4 × 4 × 1.5 m) near the inlet of an earth pond (90 × 60 × 1.5 m) in Jiaxing City, Zhejiang province, China, were fed an extruded diet without antibiotics (the feed formulation and composition were the same as the control diet described by He et al. 2009) for 1 mo and randomly divided into 2 groups (large cages). One group was cultured at the same location; the other was reared near the outlet of the pond, where fish bacterial enteritis has occurred. Both groups of fish were fed the same diet as described above. After 2 mo, fish cultured near the outlet began to die. The dominant bacteria were isolated from the GI tract of diseased fish (showing typical symptoms of bacterial enteritis) with nutritive agar (Xu et al. 2001) and identified to be *Aeromonas hydrophila* by commercial identification systems (MicroScan WalkAway system) (Rodriguez et al. 1999). However, the fish reared near the inlet seemed to be healthy and no such bacterial pathogens were identified. To heal bacterial enteritis, surviving fish from both groups were randomly subdivided into 2 subgroups (small cages 1.0 × 1.0 × 1.0 m with 12 individuals per cage within the large cage). Two subgroups (1 healthy and 1 diseased) were fed new fodder that contained 1.5 g trimethoprim/sulfamethoxazole per 1 kg diet (the ratio of trimethoprim and sulfamethoxazole was 1:5, w/w), and 2 other subgroups were fed the same extruded diet without antibiotics. After 4 d, bacterial enteritis was successfully controlled and no additional dead fish were detected. Five fish of each subgroup were randomly collected and stored at –20 °C for further analyses. The effects of antibiotics discharged from fish fed the antibiotic fodder into pond water were not considered in the present study.

Sample preparation

The fish skin was washed with 70% ethanol in order to reduce contamination. The ventral surface was opened with sterile scissors to expose the body cavity. The digestive tract was sampled, closed off with sterile clamps, and then cut free. The external surface was washed with sterile phosphate-buffered saline (PBS). The intestinal contents of the gut sections were used for DNA extraction. The whole intestine was divided into 2 sections, i.e. the foregut (defined as the region at the beginning of the GI tract) and the hindgut (the region at the end of the intestine); each section was 3 cm in length. Because gut microbiota were found to vary a lot within individuals (Liu et al. 2008), each section of 3 individual fish was aseptically opened with a sterile scalpel and pooled together to provide a representative sample (Zhou et al. 2007). A total of 8 samples were prepared, and each sample was homogenized using a glass homogenizer (LeaMaster et al. 1997) and stored in Eppendorf tubes at –20°C until analysis.

Extraction and purification of DNA

The total genomic DNA of intestinal microbiota was extracted as described by Brady (2007) with some modifications. Briefly, homogenized intestinal samples were suspended in 1 ml of 65°C preheated lysis buffer (200 mM Tris-HCl, 100 mM Na EDTA, 500 mM NaCl, 1% [w/v], 2% [w/v] sodium dodecyl sulfate [SDS], pH 8.0) in a 1.5 ml centrifuge tube, inverted to mix, and incubated in a 65°C water bath for 1 h with gentle inversion every 20 min. The tube was then removed from the water bath, cooled down to room temperature, and centrifuged at 10 000 × *g* for 10 min at 4°C. The supernatant was transferred into a fresh 1.5 ml centrifuge tube, and isopropanol was added for DNA precipitation. The genomic DNA

was purified using a Cycle-Pure DNA kit (Omega). The presence and size of purified DNA were visualized on an agarose gel by ethidium bromide staining.

PCR amplification and DGGE analysis

Universal primers BA338f (5′-ACT CCT ACG GGA GGC AGC AG-3′) with a 40 base GC clamp at the 5′ end and UN543r (5′-TTA CCG CGG CTG CTG G-3′) were used for amplification of the variable V3-region on 16S rDNA. Ten nanograms of purified genomic DNA were used as template per PCR reaction. The PCR and DGGE were performed as described previously (Liu et al. 2008). The purified PCR product (900 ng) of each sample was loaded directly onto the polyacrylamide gel (8%, w/v) with a denaturing gradient ranging from 40 to 60%. DGGE was performed with a D-Code universal mutation detection system (Bio-Rad). Electrophoresis was conducted with a constant voltage of 50 V at 60°C for about 16 h. Then the gel was stained with ethidium bromide and washed with deionized water before being photographed under UV transillumination.

Sequencing and phylogenetic analysis

DGGE profiles were scanned with Bio-1D++ software (Vilber-Lourmat). The banding patterns were carefully checked manually. Similarities between the PCR-DGGE banding patterns were analyzed using the Pearson correlation coefficient and displayed graphically as a dendrogram. An unweighted pair-group clustering algorithm with arithmetic averages (UPGMA) was used to calculate the dendrograms. A pairwise similarity coefficient (*C*s) less than 0.60 is regarded as significantly different, while $0.60 \leq Cs < 0.85$ is regarded as marginally different, and $Cs \geq 0.85$ is regarded as very similar according to Sun et al. (2004). The PCR bands were sequenced and checked for chimeric constructs by using the check chimera program of the ribosomal database project (RDP) (Cole et al. 2005). All sequences were submitted for similarity searches with the BLAST program (Altschul et al. 1990).

Nucleotide sequence accession numbers

All nucleotide sequences retrieved from the DGGE gel were deposited in the GenBank database under accession numbers HQ401026 to HQ401049.

RESULTS

Sample preparation and pathogen identification

Two gut samples (1 from the foregut and 1 from the hindgut) were collected from each of the following 4 subgroups: healthy fish with a regular diet (A); diseased fish with a regular diet (B); healthy fish with an antibiotic diet (C); diseased fish with an antibiotic diet (D). *Aeromonas hydrophila*, which has been identified to be the causative agent of fish disease (Chu & Lu 2000), was found in the gut of the infected fish.

Bacterial community

All of the digestive tract samples gave clear PCR products after amplification with primers BA338f and UN543r specific for the V3 region of bacterial 16S rRNA gene sequences (data not shown). The 16S rDNA V3 PCR–DGGE fingerprints of the 8 samples are shown in Fig. 1. A total of 26 bands were identified, and each band is theoretically equivalent to 1 unique bacterial operational taxonomic unit (OTU)

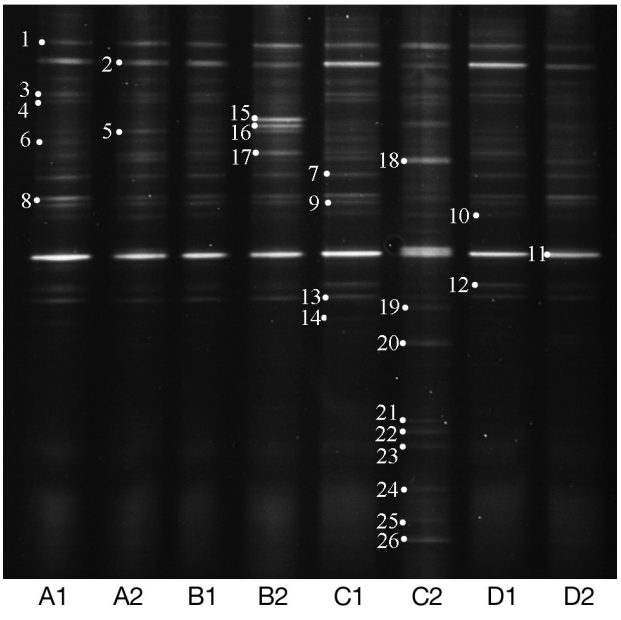

Fig. 1. DGGE profiles generated from PCR-amplified V3-16S rRNA gene obtained from genomic DNA extracted from fish samples. A1: healthy fish foregut without antibiotic treatment; A2: healthy fish hindgut without antibiotic treatment); B1: diseased fish foregut without antibiotic treatment; B2: diseased fish hindgut without antibiotic treatment; C1: healthy fish foregut with antibiotic treatment; C2: healthy fish hindgut with antibiotic treatment; D1: diseased fish foregut with antibiotic treatment; D2: diseased fish hindgut with antibiotic treatment

(Muyzer et al. 1993). The average number of bands was 17 (range from 15 to 23). The banding pattern noted in position, intensity and number was relatively invariant before and after antibiotic exposure. The PCR–DGGE fingerprint of the 16S rDNA V3 region of the bacteria from different samples was further analyzed based on dendrograms (Table 1, Fig. 2). Without exposure to antibiotics, the bacterial communities of Samples A1 (healthy fish foregut), A2 (healthy fish hindgut), and B1 (diseased fish foregut) were confined to one cluster. Sample B2 (diseased fish hindgut) without exposure to antibiotics showed variation from the other samples. Those samples with antibiotic treatment were clustered together, including C1 (healthy fish foregut), C2 (healthy fish hindgut), D1 (diseased fish foregut), and D2 (diseased fish hindgut). Samples from the same gut position of the 4 groups had higher similarities than those with different positions from the same group. UPGMA analysis showed the relationship of bacterial communities between 2 gut positions (foregut and hindgut) and 4 treatments (healthy fish with a regular diet, diseased fish with a regular diet, healthy fish with an antibiotic diet and diseased fish with an antibiotic diet). The bacterial communities of Samples A1 and A2 were more similar (0.71) than those of Samples B1 and B2 (0.67). With exposure to antibiotics, the similarities of bacterial communities of Samples C1 and C2 (0.29) and that of Samples D1 and D2 (0.46) were low. However, the bacterial communities of Samples C1 and D1 (0.71) and Samples C2 and D2 (0.77) showed high similarities.

Analysis of bacterial 16S rRNA gene

In order to determine the bacterial group or taxon of particular bands, 26 distinct bands were excised from the DGGE gel for sequencing. Only 24 bands were successfully sequenced and compared with the NCBI database (Table 2). *Actinobacteria*, *Firmicutes* and *Proteobacteria* were the predominant phyla, but the bacterial types varied a lot among samples. Some bands became intensified, disappeared or appeared after antibiotic exposure. Bands 15–17 disappeared or weakened, and Bands 18–26 intensified or appeared. The bands that seemed unaffected by antibiotic exposure all belonged to *Gammaproteobacteria*.

Table 1. Pairwise similarity coefficients (*C*s) matrix for intestinal bacterial communities of 8 samples. See Fig. 1 for sample abbreviations

	A1	A2	B1	B2	C1	C2	D1	D2
A1	1.00							
A2	0.71	1.00						
B1	0.88	0.71	1.00					
B2	0.40	0.46	0.67	1.00				
C1	0.53	0.46	0.67	0.57	1.00			
C2	0.27	0.31	0.27	0.43	0.29	1.00		
D1	0.40	0.31	0.53	0.57	0.71	0.43	1.00	
D2	0.43	0.33	0.43	0.46	0.46	0.77	0.46	1.00

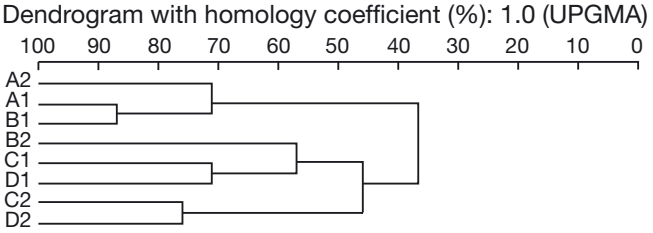

Fig. 2. Dendrogram generated from DGGE profiles of intestinal samples, representing similarities and variations in banding pattern among groups. See Fig. 1 for sample abbreviations

DISCUSSION

This is the first time that the effect of treatment with antibiotics on the intestinal microbiota of fish has been determined using PCR–DGGE technology. *Actinobacteria*, *Firmicutes* and *Proteobacteria* were determined to be the predominant phyla in healthy untreated gibel carp intestine. The same result has been reported by previous studies that employed *in situ* hybridization (FISH) (Asfie et al. 2003, Nayak 2010).

The antibiotic effect was mainly observed in the bacterial communities of the hindgut of diseased fish (Table 1, Fig. 2). Compared with healthy fish, the ecological equilibrium of the intestinal microbiota of diseased fish had been changed by the presence of bacterial enteritis pathogens. A previous study has indicated that pathogens present in the transient state can break the balance and establish lethal infections (Sekirov & Finlay 2009). Based on sequence data, we found that in diseased fish Bands 15–17 disappeared or weakened after successful antibiotic treatment. These bands are closely related with *Aeromonas* sp. A514A (Band 15), *Enterobacter* sp. VF20 (2010) (Band 16) and *Enterobacter* sp. (Band 17). Some *Aeromonas* spp. have been reported as the etiologic agents of GI infection of fish, especially *A. hydrophila,* which is considered a major economic problem for the fish-farming industry (Austin & Austin 1999). In this study, we identified the presence of *A. hydrophila* in the diseased fish group. In

Table 2. Closest nucleotide sequences matched to excised DGGE bands (1–26). See Fig. 1 for sample abbreviations

Phylogenetic group	Band no.	Closest relative (obtained from BLAST search)	Identity (%)	GenBank accession no.	Relative abundance (%) A1	A2	B1	B2	C1	C2	D1	D2
Clostridia												
	19	*Clostridium sordellii* (HM163489)	100	HQ401042						2.7		
Actinobacteria												
	14	*Actinobacterium* (GQ222445)	100	HQ401037	1.3		0.9		1.1		1	
	18	Uncultured actinobacterium (EF446353)	100	HQ401041						10.7		3.3
	21	Uncultured actinobacterium (AB265918)	98	HQ401044						2.4		1.4
	22	Uncultured actinobacterium (DQ828649)	100	HQ401045						3.2		
	23	*Streptomyces carpaticus* (HQ132783)	100	HQ401046						1.6		
	25	Uncultured actinobacterium (HM153633)	100	HQ401048						5.9		2.3
	26	*Actinobacterium* (FJ460072)	100	HQ401049						0.7		
Gammaproteobacteria												
	1	*Acinetobacter* sp. (EU267627)	100	HQ401026	4.4	7.5	6.9	7.1	6.5	8.4	6.7	4.8
	2	*Acinetobacter junii* (FN811324)	100	HQ401027	11.9	10.3	13.0	5.7	15.5	3.2	21.0	9.2
	3	*Acinetobacter baylyi* (FJ009376)	100	HQ401028	1.9	2.9	2.1	2.1	2.4	2.7	1.8	2.7
	5	*Salmonella* sp. (EU682263)	99	HQ401029	1.1	4.4	1.8	3.1				
	7	*Enterobacter* sp. (EU438989)	99	HQ401030	5.6	4.1	5.7	4.3	4.2		4.6	4.2
	8	*Salmonella* sp. (FJ463830)	100	HQ401031	8.7	3.1	5.0	3.2	4.8	2.3	3.0	8.2
	9	*Enterobacter* sp. (AB461711)	99	HQ401032	5.6	3.3	4.1	3.1	4.3		2.9	4.2
	10	*Enterobacter* sp. (AB461711)	100	HQ401033	3.2	4.2	1.3	1.6	2.0	1.8	3.3	2.6
	11	*Enterobacter cloacae* (HQ154578)	100	HQ401034	36.8	40.7	39.9	28.0	32.3	33.4	32.5	37.8
	12	*Enterobacter* sp. (HM461187)	100	HQ401035	3.7	1.9	2.6	1.6	3.8	0.5	4.0	2.5
	13	*Enterobacter* sp. (AF321020)	100	HQ401036	4.9	3	4.2	3.9	5.8	0.9	4.1	3.5
	15	*Aeromonas* sp. (HQ122921)	100	HQ401038				12.4				
	16	*Enterobacter* sp. (2010) (HQ014909)	98	HQ401039				11.2		5.3		4.3
	17	*Enterobacter cloacae* (HM162426)	99	HQ401040	2.8	7	4.3	8.3	5.4		7.2	
Alphaproteobacteria												
	20	*Blastochloris sulfoviridis* (AY117150)	99	HQ401043						5.9		3.3
	24	Methylobacteriaceae bacterium (FJ005068)	98	HQ401047						2.5		
Unsuccessfully sequenced band												
	4				7.2	5.7	8.1	4	7.9	5.1	6.5	4.0
	6				1.6	1.9	1.2	0.4	3.9		1.5	1.6

addition to *Aeromonas*, several enteroinvasive members of *Enterobacter* also can infect fish (Baldwin & Newton 1993). Based on the results mentioned above, which agree with bacterial species previously identified as causative agents of gut enteritis in fish, PCR–DGGE technology was proved to be a rapid and reliable approach to monitor the diversity and dynamics of intestinal microbiota and to identify pathogens for efficient control.

The bacterial community structures of the different samples were closely related after the trimethoprim/sulfamethoxazole treatment, especially those between Samples C1 and D1 (0.71), and Samples C2 and D2 (0.77). This result agrees with our previous study in that the similarity of microbiota in specific sections along the GI tract of different fish was higher than the similarity of different sections along the GI tract of the same fish (Zhou et al. 2009). The lower similarities of bacterial community structures of Samples C1 and C2 (0.29) and Samples D1 and D2 (0.46), compared to similarities between the fore- and hindgut in untreated (diseased and healthy) fish, suggested that the microfauna of fore- and hindgut were differentially affected by the antibiotics used here. However, Sample B2 (diseased fish hindgut) without contact with antibiotics clustered with those with the antibiotic treatment, which may be caused by the bacterial enteritis. A previous study indicated that the process of pathogenic infection results in a disturbance to the microbiota (Sekirov & Finlay 2009). Our result also indicated that the diseased fish hindgut is the main site of the bacterial enteritis outbreak. More changes in the bacterial community structures in healthy fish hindgut treated with antibiotics were identified than in the diseased fish, suggesting that antibiotic treatment has a greater impact on healthy fish. The GI tract is a favorable ecologic niche for a wide range of microbes (Nayak 2010). However, if the dominant bacterial species in the healthy fish are inhibited by antibiotics, then previ-

ously non-dominant bacterial species may become dominant. Therefore, antibiotics also promote more bacterial species (23 bands in healthy treated groups vs. <20 bands in other groups; Fig. 1 & Table 2) under the selective pressure in healthy fish.

There were no obvious changes in the fore- and hindguts of all groups in Bands 1, 3, 7, 8, 9, 11 and 13 based on the PCR–DGGE fingerprint. Bands 5 and 12 revealed slight changes in healthy fish gut after the antibiotic treatment, both bands showing high similarities to *Enterobacteriaceae*. Van der Veen et al. (2009) found that trimethoprim/sulfamethoxazole was closely associated with the appearance of (multi)drug-resistant *Enterobacteriaceae* in child intestinal flora. *Enterobacteriaceae* might be the major drug-resistant bacteria in gibel carp intestine. Bands 1–3 in both the hind- and foregut showed similarity to *Acinetobacter*, which are non-fermentative organisms and frequently susceptible to trimethoprim/sulfamethoxazole (Fass et al. 1996). However, in this study Bands 1–3 showed no obvious changes after antibiotic treatment, which might be ascribed to resistance selection. Furthermore, Bands 5 and 8 from both gut positions in healthy and diseased fish fed a regular diet, which were similar to *Salmonella* sp., might also be resistant to trimethoprim/sulfamethoxazole. *Salmonella* has been found in contaminated fish feed. In an aquaculture operation study of freshwater fish in Guangdong Province, China (Broughton & Walker 2009), *Salmonella* sp. was isolated and showed susceptibility to neomycin, cefotaxime and cefepime but was resistant to erythromycin and penicillin. After antibiotic treatment, Bands 18–26 appeared or intensified, suggesting changes in the GI microbiota diversity. Antibiotic treatment might cause some non-dominant bacterial species to become the dominant ones.

Because the majority of the GI tract bacteria are uncultivable (Zoetendal et al. 2008), bacterial count information obtained by traditional methods is always limited. The PCR–DGGE approach allows identification of the predominant species and the characterization of the changes in community composition. The disadvantage is that this approach fails to reflect the actual total diversity of any given sample.

CONCLUSIONS

This study has shown the changes in the bacterial community composition of gibel carp with bacterial enteritis and in the bacterial community of gibel carp GI following treatment with the antibiotics trimethoprim and sulfamethoxazole. The antibiotic treatment changed the gut bacterial communities in healthy and recovered fish, and the effect and duration of such changes need to be investigated further.

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