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Short communication

Phenotypic and genotypic characterisation of antimicrobial resistance in faecal bacteria from 30 Giant pandas

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ABSTRACT

To study the prevalence of antimicrobial resistance in faecal bacteria from Giant pandas in China, 59 isolates were recovered from faecal pats of 30 Giant pandas. Antimicrobial susceptibility testing of the isolates was performed by the standardised disk diffusion method (Kirby–Bauer). Of the 59 study isolates, 32.20% were resistant to at least one antimicrobial and 16.95% showed multidrug-resistant phenotypes. Thirteen drug resistance genes [*aph(3′)-IIa*, *aac(6′)-Ib*, *ant(3″)-Ia*, *aac(3)-IIa*, *sul1*, *sul2*, *sul3*, *tetA*, *tetC*, *tetM*, *cat1*, *floR* and *cmlA*] were analysed using four primer sets by multiplex polymerase chain reaction (PCR). The detection frequency of the *aph(3′)-IIa* gene was the highest (10.17%), followed by *cmlA* (8.47%). The genes *aac(6′)-Ib*, *sul2* and *tetA* were not detected. PCR products were confirmed by DNA sequence analysis. The results revealed that multidrug resistance was widely present in bacteria isolated from Giant pandas.

1. Introduction

The Giant panda, *Ailuropoda melanoleuca*, is one of the most endangered and rare animals in the world. It used to be widely distributed in southwest China, including Hunan, Hubei, Sichuan, Shanxi and Gansu provinces, in the 16th to 19th centuries, but today it only occurs in Sichuan, Shanxi and Gansu provinces [1].

Although some Giant panda conservation programmes have been initiated, the species is still facing the crisis of extinction because of their low reproductive rate, high specialisation for food, poor resistance to diseases and habitat destruction [2]. Above all, intestinal tract disease caused by pathogenic bacteria is a significant threat to the health of Giant pandas, and antimicrobials have proved a useful way of preventing and curing infectious diseases of the Giant panda.

However, it must be recognised that one of the foremost challenges in the management of infectious diseases is antimicrobial resistance, which is a serious problem worldwide. Moreover, the problem becomes more and more acute year by year [3]. Widespread antibiotic use has accelerated the development and spread of antibiotic- and multidrug-resistant (MDR) bacteria in humans and the environment. Especially in recent years, antimicrobial resistance in bacteria of animal origin has drawn much attention worldwide [4].

Antimicrobial susceptibility testing and resistance gene investigation is needed to provide valuable information in order to understand the epidemiology of antimicrobial resistance. Usually, conventional susceptibility testing methods are time consuming and have limitations in terms of generating false-positive and false-negative results. Polymerase chain reaction (PCR)-based methods are regarded as more convenient to detect resistance [5]. Most of the established methods for detecting drug resistance are based on a single gene by standard PCR. To overcome the inherent disadvantage of cost and to improve the diagnostic capacity of the test, multiplex PCR, a variant of the test in which more than one target sequence is amplified using more than one pair of primers, has been developed and is widely used in clinical diagnosis as well as in drug resistance gene detection [6].

To the best of our knowledge, little research has been done to investigate the antimicrobial resistance phenotypes and genotypes of isolates from faecal samples of Giant pandas. Therefore, the objectives of the present study were to characterise the antimicrobial resistance phenotypes and genotypes among Giant pandas' faecal sample isolates as well as their correlation.

2. Materials and methods

2.1. Bacterial strains

Fifty-nine isolates were collected from faecal pats of 30 Giant pandas living in the China Research and Conservation Center for the Giant Panda, Wolong Nature Reserve, Sichuan, China. All of

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the isolates were presumptively identified by phenotypic methods, including colony morphology on agar, Gram stain and growth on selective culture medium. Identification was later confirmed by the Vitek system (bioMérieux, Durham, NC). The isolates were *Escherichia coli* ($n = 38$), *Enterobacter aerogenes* ($n = 3$), *Salmonella* sp. ($n = 5$), *Pseudomonas aeruginosa* ($n = 5$), *Streptococcus faecalis* ($n = 3$) and *Staphylococcus* sp. ($n = 5$). All of the isolates were stored in Luria–Bertani broth containing 15% glycerol at −80 °C until used.

2.2. Antimicrobial susceptibility testing

The susceptibilities of all the isolates to 10 antimicrobials were determined using the standard Kirby–Bauer disk diffusion method. The following antimicrobial disks from Oxoid Ltd. (Basingstoke, UK) were used: kanamycin (30 μg); streptomycin (10 μg); gentamicin (10 μg); amikacin (30 μg); sulfamethoxazole (25 μg); sulfamethoxazole/trimethoprim (1.25/23.75 μg); tetracycline (30 μg); doxycycline (30 μg); chloramphenicol (30 μg); and florfenicol (30 μg). All isolates were inoculated into Mueller–Hinton (MH) broth (Becton Dickinson, Franklin Lakes, NJ) and incubated overnight at 37 °C. The suspensions were adjusted to the turbidity of a 0.5 McFarland standard and streaked onto MH agar plates. Antimicrobial disks were placed on the plates, which were incubated aerobically at 35 ± 2 °C for 16–18 h. The diameter of the inhibition zones surrounding the antimicrobial disks was interpreted according to Clinical and Laboratory Standards Institute guidelines. Quality control for susceptibility testing was done using *E. coli* ATCC 25922.

2.3. Detection of resistance genes

2.3.1. Primers

The design of DNA oligonucleotide primers used for PCR amplification was based on the published genome sequence in the GenBank database using Primer Premier Software 5.0 (Premier Biosoft International, Palo Alto, CA). Synthesis of oligonucleotides was carried out at Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). For multiplex PCRs, four primers sets were prepared (Table 1): set A was designed to amplify aminoglycoside resistance genes [*aph(3′)-IIa*, *aac(3)-IIa*, *aac(6′)-Ib* and *ant(3″)-Ia*]; set B was designed to amplify sulfonamide resistance genes (*sul1*, *sul2* and *sul3*); set C was designed to amplify tetracycline resistance genes (*tetA*, *tetC* and *tetM*); and set D was designed to amplify chloramphenicol resistance genes (*cat1*, *cmlA* and *floR*).

2.3.2. Bacterial DNA preparation and PCR amplification

All DNA templates were prepared by the boiling method [7]. The amount of each primer pair used in the assay was first optimised to achieve maximum amplification in separate reactions using the standard PCR programme and reaction conditions. Next, the amount of each primer pair in different multiplex PCR sets was balanced to achieve acceptable amplification of all target genes. For each set of multiplex PCR reactions, a standard 25 μL reaction mixture was used. The reagents for each group were as follows: 10 μmol/L primers [Set A, *aph(3′)-IIa* (0.5 μL), *aac(3)-IIa* (0.9 μL), *aac(6′)-Ib* (0.8 μL), *ant(3″)-Ia* (1.8 μL); Set B, *sul1* (1.4 μL), *sul2* (0.4 μL), *sul3* (1.2 μL); Set C, *tetA* (1.0 μL), *tetC* (0.3 μL), *tetM* (0.4 μL); and Set D, *cat1* (0.2 μL), *floR* (0.4 μL), *cmlA* (0.9 μL)]; 2.5 μL of Mg^{2+}-free 10 × PCR reaction buffer; 2 μL of 2.5 μmol/L $MgCl_2$; 1.0 U *Taq* polymerase mix; 2 μL of 250 μmol/L deoxyribonucleotide mix; 5 μL of DNA solution containing 50 ng of DNA template; and an appropriate amount of PCR-grade water to make a total volume of 25 μL. The thermocycling parameters included an initial denaturing step at 94 °C for 5 min, 35 cycles of 94 °C for 50 s, 54 °C for 50 s and 72 °C for 60 s and a final extension at 72 °C for 10 min. The amplified products were separated by gel electrophoresis in a 2.0% agarose gel stained with GoldView™ (Shanghai SBS Genetech Technology Co., Ltd., Shanghai, China), visualised under ultraviolet light and recorded using a gel documentation system (Bio-Rad, Hercules, CA).

2.4. Nucleotide sequence analysis

PCR products ($n = 12$) were purified using the QIAquick PCR Purification Kit (QIAGEN Inc., Valencia, CA) and were sequenced by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. on both strands by automated sequencing with an ABI Prism 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA). The resulting DNA sequence data were compared with data in the GenBank database using the BLAST algorithm available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov).

3. Results

3.1. Susceptibility of all the isolates

The antimicrobial susceptibility patterns of all the isolates are presented in Tables 2 and 3; 32.20% of the isolates ($n = 19$) were resistant to at least one antimicrobial and 16.95% ($n = 10$) were resistant to three or more antimicrobials (i.e. MDR). Strain P14 (*Staphylococcus* sp.) was the most resistant isolate in the study,

Table 1
Primers used in the study.

Multiplex PCR sets	Target genes	Oligonucleotide primer sequences		Product size (bp)	Accession no.
		Forward primer (5′→3′)	Reverse primer (5′→3′)		
Set A	*aph(3′)-IIa*	TGACTGGGCACAACAGACAA	CGGCGATACCGTAAAGCAC	677	AY222814
	aac(3)-IIa	ACCCTACGAGGAGACTCTGAATG	CCAAGCATCGGCATCTCATA	384	X51534
	aac(6′)-Ib	ATGACCTTGCGATGCTCTATGA	CGAATGCCTGGCGTGTTT	486	AJ009820
	ant(3″)-Ia	ATCTGGCTATCTTGCTGACA	TATGACGGGCTGATACTGG	284	AF282595
Set B	*sul1*	CATTGCCTGGTTGCTTCAT	ATCCGACTCGCAGCATTT	238	AB061794
	sul2	CATCATTTTCGGCATCGTC	TCTTGCGGTTTCTTTCAGC	793	NC005324
	sul3	AGATGTGATTGATTTGGGAGC	TAGTTGTTTCTGGATTAGAGCCT	443	AY316203
Set C	*tetA*	GGCACCGAATGCGTATGAT	AAGCGAGCGGGTTGAGAG	480	X75761
	tetC	CTGGGCTGCTTCCTAATGC	AGCTGTCCCTGATGGTCGT	580	J01749
	tetM	GAGGTCCGTCTGAACTTTGCG	AGAAAGGATTTGGCGGCACT	900	DQ534550
Set D	*cat1*	AACCAGACCGTTCAGCTGGAT	CCTGCCACTCATCGCAGTAC	550	NC003384
	floR	GGCTTTCGTCATTGCGTCTC	ATCGGTAGGATGAAGGTGAGGA	650	AF261825
	cmlA	TGCCAGCAGTGCCGTTTAT	CACCGCCCAAGCAGAAGTA	900	AJ487033