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Detection of Multi-Drug-Resistant *Escherichia coli* in a Giant Panda (*Ailuropoda melanoleuca*) with Extraintestinal Polyinfection

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ABSTRACT: Multi-drug-resistant *Escherichia coli*, *Pseudomonas aeruginosa*, and *Enterococcus faecalis* were isolated from the tissue samples of a giant panda. The *E. coli* strain SH-YH-DH that we isolated carried a self-transferable plasmid associated with multi-drug-resistant genes ($bla_{CTX\text{-}M\text{-}55}$, $bla_{TEM\text{-}1}$, *sul1*, *floR*, *strB*, *aac(6′)-Ib*, and *tetA/R*). This strain exhibited phenotypic resistance to gentamicin, cefotaxime, ampicillin, chloramphenicol, trimethoprim-sulfamethoxazole, and tetracycline. We report the participation of multi-drug-resistant *E. coli* in a extraintestinal polyinfection in giant pandas and that further research is warranted to pay attention to the threat of multi-drug-resistant bacteria in the panda population.

On 26 December 2014, a male 3-yr-old giant panda in Shanghai Wild Animal Park (Shanghai, China) became anorexic and developed a fever. No blood testing was done at the time. He was treated intravenously with adenosine triphosphate, sodium selenite-vitamin E, and glucose but not with antibiotics until 30 December 2014. Sulfamethoxazole-trimethoprim was started on 1 January 2015, but the panda died on 2 January 2015. He had a history of infection with *Ascaris lumbricoides*, and he weighed only 60 kg, much less than the average body weight of more than 80 kg that is typical for a 3-yr-old male giant panda.

Tissue samples (heart, liver, spleen, lung, kidney, and mesenteric lymph nodes) were collected from the dead giant panda by two trained individuals. The samples were stored independently in ice boxes and immediately transported to our laboratory in Changchun. General analytical methods were performed, including microscopic observation after Gram staining, and colony morphology after direct culturing on 5% sheep blood agar (Oxoid, Wesel, Germany) incubated at 37 C for 24 h under aerobic conditions. All manipulations were conducted in a biosafety cabinet.

Based on the colony appearance (diameter, color, edge morphology, and hemolysis) on blood agar, nine isolates from each sample (27 in total, since three samples yielded no isolate; Table 1) were selected and subcultured on lysogeny broth agar (Oxoid, Hampshire, UK), and lawns were collected and resuspended in 5 mL sterile distilled water. We prepared DNA templates from 1 mL bacterial suspensions using a DNA extraction kit (QIAamp-DNA Mini Kit, Qiagen, Valencia, California, USA) according to the manufacturer's instructions. Enterobacterial repetitive intergenic consensus PCR was conducted to assess the relationship between isolates based on their DNA fingerprints (James et al. 1991), and isolates were identified by 16S ribosomal DNA gene sequencing (Weisburg et al. 1991). Further confirmation of the bacterial species and antimicrobial susceptibility testing were done using a BD Phoenix 100 automated microbiology system (Becton, Dickinson and Company, Sparks, Maryland, USA).

Three different colony morphologies were apparent among the nine colonies selected from blood agar plates from lung tissue and two different morphologies among the nine colonies from spleen and mesenteric lymph nodes samples. The 27 isolates were divided into three groups: Group 1 contained 13 Gram-negative isolates (three from lung, five from spleen, and five from mesenteric lymph nodes), Group 2 contained seven Gram-negative isolates (three from lung and four from spleen), and Group 3 contained seven Gram-positive isolates (three from lung and four from mesenteric lymph nodes). Isolates in the same group showed the same DNA fingerprint. The three species were confirmed

TABLE 1. Isolates (n=27) of *Escherichia coli*, *Pseudomonas aeruginosa*, and *Enterococcus faecalis* made from tissues taken from a captive giant panda (*Ailuropoda melanoleuca*) that died from multiple infections on 2 January 2015 in the Republic of China. No isolates were made from hearts, livers, and kidneys.

Tissues	No. colony types	Colony morphology	Isolate designation (strain identifier)	Species	Fingerprint type (group)	No. per group
Spleen	2	Diameter 1.0–1.5 mm, milky-white, edge tidy, smooth appearance and nonhemolytic	SH-YH-DH	*E. coli*	1	5
		Diameter 1.5–2.5 mm, gray-white, edge irregularity, rough appearance and β-hemolytic	SH-YH-DB	*P. aeruginosa*	2	4
Lung	3	Diameter 1.0–1.5 mm, milky-white, edge tidy, smooth appearance and nonhemolytic	SH-YH-DH	*E. coli*	1	3
		Diameter 1.5–2.5 mm, gray-white, edge irregularity, rough appearance and β-hemolytic	SH-YH-DB	*P. aeruginosa*	2	3
		Diameter 0.5–1.0 mm, yellowish-white, edge tidy, smooth appearance and nonhemolytic	SH-YH-XB	*E. faecalis*	3	3
Mesenteric lymph nodes	2	Diameter 1.0–1.5 mm, milky-white, edge tidy, smooth appearance and nonhemolytic	SH-YH-DH	*E. coli*	1	5
		Diameter 0.5–1.0 mm, yellowish-white, edge tidy, smooth appearance and nonhemolytic	SH-YH-XB	*E. faecalis*	3	4

TABLE 2. Minimum inhibitory concentrations (MICs) of antibiotics for the *Escherichia coli* strain SH-YH-DH and transconjugant. The MICs were identical for both the *E. coli* SH-YH-DH and the transconjugate. The *E. coli* strain SH-YH-DH was isolated from a captive giant panda (*Ailuropoda melanoleuca*) that died from multiple infections on 2 January 2015 in the Republic of China.

	MIC (mg/L)		
Antibiotic	Automated method[a]	Agar dilution method[b]	Resistance
Amikacin	<8	—	Susceptible
Gentamicin	>8	64	Resistant
Imipenem	<1	—	Susceptible
Meropenem	<1	—	Susceptible
Cefazolin	>16	—	Resistant
Ceftazidime	8	32	Resistant
Cefotaxime	>32	256	Resistant
Cefepime	>16	256	Resistant
Aztreonam	>16	128	Resistant
Ampicillin	>16	256	Resistant
Piperacillin	>64	—	Resistant
Amoxicillin-clavulanate	8/4	—	Susceptible
Ampicillin-sulbactam	16/8	—	Intermediate
Piperacillin-tazobactam	<4/4	—	Susceptible
Colistin	<0.5	—	Susceptible
Trimethoprim-sulfamethoxazole	>2/38	—	Resistant
Chloramphenicol	>16	256	Resistant
Ciprofloxacin	<0.5	—	Susceptible
Levofloxacin	<1	—	Susceptible
Tetracycline	>8	256	Resistant

[a] Minimum inhibitory concentrations are based on the BD automated microbiology system Phoenix 100 (Becton Davidson, Sparks, Maryland, USA).

[b] Minimum inhibitory concentrations are based on the agar dilution method following the standards (M100S) of the Clinical Laboratory Standards Institute (2016). — = not done.

as *Escherichia coli* (SH-YH-DH), *Pseudomonas aeruginosa* (SH-YH-DB), and *Enterococcus faecalis* (SH-YH-XB), based on the 16S ribosomal DNA sequencing and biochemical test results (Table 1).

Based on the antimicrobial susceptibility tests using the automated microbiology system, the *E. coli* isolate SH-YH-DH attracted our attention due to its multiple-drug resistance phenotype. The minimum inhibitory concentrations (MICs) for the antibiotics gentamicin, ceftazidime, cefotaxime, cefepime, aztreonam, ampicillin, chloramphenicol, and tetracycline were determined by the agar dilution method on Mueller-Hinton agar according to guideline M100S of the Clinical and Laboratory Standards Institute (2016). We used *E. coli* (ATCC 25922) as a quality control strain.

Escherichia coli SH-YH-DH showed high-level resistance to cefotaxime, cefepime, aztreonam, ampicillin, chloramphenicol and tetracycline, gentamicin, and ceftazidime (Table 2). The resistance genes were determined by PCR (Dallenne et al. 2010) and sequencing. The $bla_{\text{CTX-M-55}}$, $bla_{\text{TEM-1}}$, *sul1*, *floR*,

strB, *aac(6′)-Ib*, and *tetA/R* genes were detected in *E. coli* SH-YH-DH.

Conjugation experiments were conducted by filter-mating using *E. coli* SH-YH-DH as the donor and *E. coli* J53 Azi^R as the recipient (Jacoby and Han 1996). The viable cell count in the suspension was 10^9 colony-forming-units/mL, 113 colonies grew on the resistant medium, and the transfer frequency was 10^{-6}. The positive transconjugant was obtained from *E. coli* SH-YH-DH, and it acquired the same resistance genes and showed a similar resistance phenotype as did *E. coli* SH-YH-DH (Table 2), with MICs for gentamicin, ceftazidime, and aztreonam that were only slightly lower than the MICs for *E. coli* SH-YH-DH.

Plasmids from SH-YH-DH and transconjugants were extracted using a plasmid midi kit (Qiagen, Shanghai, Republic of China) and used as templates for PCR-based replicon typing (Johnson et al. 2007) and plasmid double-locus sequence typing (Multi Locus Sequence Typing 2010). Replicon typing revealed the presence of the IncHI2 replicon in *E. coli* SH-YH-DH and transconjugants, and pDLST analysis of the ST of pSH-YH-DH from transconjugants led to assignment as *E. coli* ST3-IncHI2 (GenBank accession no. KX129949).

The multi-drug-resistant (MDR) *E. coli* strain SH-YH-DH was isolated from tissue samples from a dead panda coinfected with *P. aeruginosa* and *E. faecalis*. The cause of the panda's death was considered to be multiple infections. Based on the plasmid-mediated MDR genes, *E. coli* SH-YH-DH showed high-level resistance to antibiotics that might influence antibiotic treatments. We carried out the characterizations of the bacteria that we isolated as clinically relevant. Multi-drug-resistant *E. coli* have been reported only rarely in pandas (Zou et al. 1998; Wang et al. 2015).

In 2015, a total of 2,286 pandas were living in China, including 1,864 wild individuals and 422 captive individuals (State Forestry Administration of China 2016). Cefazolin, amikacin, and amoxicillin-clavulanate were used for the treatment of bacterial infections in giant pandas in China (Ren et al. 2017), and treatment might have caused bacterial resistance in the diseased individual (Zhang et al. 2009). The reintroduction of giant pandas is in progress. If the captive individuals acquire drug-resistant bacteria, especially plasmid-mediated MDR bacteria, either during domestication or rescue, this could accelerate the spread of resistant bacteria through wild populations via defecation and mating (Ren et al. 2017). This could clearly have a disastrous effect on wild giant panda populations in the future, and we should therefore not overlook the prevalence of drug-resistant bacteria in this species.

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