Serotypes, Virulence Factors, and Antimicrobial Susceptibilities of Vaginal and Fecal Isolates of *Escherichia coli* from Giant Pandas

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Although *Escherichia coli* typically colonizes the intestinal tract and vagina of giant pandas, it has caused enteric and systemic disease in giant pandas and greatly impacts the health and survival of this endangered species. In order to understand the distribution and characteristics of *E. coli* from giant pandas, 67 fecal and 30 vaginal *E. coli* isolates from 21 giant pandas were characterized for O serogroups, phylogenetic groups, antimicrobial susceptibilities, and pulsed-field gel electrophoresis (PFGE) profiles. In addition, these isolates were tested for the presence of extraintestinal pathogenic *E. coli* (ExPEC) and diarrheagenic *E. coli* (DEC) by multiplex PCR detection of specific virulence genes. The most prevalent serogroups for all *E. coli* isolates were O88, O18, O167, O4, and O158. ExPEC isolates were detected mostly in vaginal samples, and DEC isolates were detected only in fecal samples. Phylogenetic group B1 predominated in fecal isolates, while groups B2 and D were frequently detected in vaginal isolates. Resistance to trimethoprim-sulfamethoxazole was most frequently observed, followed by resistance to nalidixic acid and tetracycline. All except five isolates were typeable by using XbaI and were categorized into 74 PFGE patterns. Our findings indicate that panda *E. coli* isolates exhibited antimicrobial resistance, and potentially pathogenic *E. coli* isolates were present in giant pandas. In addition, these *E. coli* isolates were genetically diverse. This study may provide helpful information for developing strategies in the future to control *E. coli* infections of giant pandas.

The giant panda or panda (*Ailuropoda melanoleuca*) is one of the most endangered and rare animals in the world. Today, it lives only in the Sichuan, Shaanxi, and Gansu provinces in China (1). The leading cause of death of pandas is various diseases, of which enteric disease is the most common. Although *Escherichia coli* is the most common cause of enteric diseases in panda, other pathogens include *Klebsiella* spp., *Campylobacter jejuni*, *Pseudomonas aeruginosa*, *Yersinia enterocolitica*, and *Clostridium welchii*. These enteric disorders seriously affect the digestion and absorption of food, compromise the immune system, and even cause serious complications and death, which endanger the survival of giant pandas (2, 3).

E. coli is an important human and animal pathogen worldwide. According to distinct virulence determinants and pathogenic features, strains of *E. coli* are classified into three main categories: commensal *E. coli*, diarrheagenic *E. coli* (DEC) (also called intestinal pathogenic *E. coli*), and extraintestinal pathogenic *E. coli* (ExPEC) (4). Intestinal pathogenic *E. coli* strains typically elicit diarrheal symptoms, while ExPEC strains cause urinary tract infections (UTIs), sepsis, abdominal infections, meningitis, cellulitis, osteomyelitis, and wound infections (5).

Based on phylogenetic backgrounds, the *E. coli* population can be classified into 4 major phylogroups (groups A, B1, B2, and D) (6). ExPEC strains belong mainly to groups B2 and D, while most commensal isolates belong to groups A and B1. Strains of groups B2 and D often carry virulence factors that are lacking in group A and B1 strains (7).

Antimicrobial therapy is an available tool for treating bacterial infections in both humans and animals. However, the broad use of antimicrobials selects for resistant bacteria, and antimicrobial-resistant pathogens result in higher morbidity and mortality rates in animals (8). Therefore, monitoring of bacterial pathogens such as

E. coli for antimicrobial resistance may provide useful information for the control and treatment of infections.

E. coli typically harmlessly colonizes the intestinal tract and vagina of giant pandas, although several *E. coli* clones can cause a variety of diseases within the intestinal tract and elsewhere in the panda under certain conditions. Enterotoxigenic *E. coli* O152 has been reported to cause hemorrhagic enterocolitis and death in pandas (3). *E. coli* has been associated with systemic sepsis (9). *E. coli* strains from various animal species have been investigated extensively (10–14). However, little is known about the distribution and characteristics of *E. coli* in giant pandas. Therefore, we carried out this study to determine serogroups, phylogenetic groups, antimicrobial susceptibilities, and pulsed-field gel electrophoresis (PFGE) profiles of 97 *E. coli* strains from 21 giant pandas. Moreover, the presence of virulence genes used to define pathogenic groups of pathogenic *E. coli* was also investigated.

MATERIALS AND METHODS

Bacterial strains and serotyping. A total of 97 *E. coli* isolates, including 67 isolates from fecal samples and 30 isolates from vaginal secretion samples, were collected from 21 healthy female giant pandas living in the Bifengxia Giant Panda Base in Sichuan Province, China, during two periods: from

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April to May 2010 and from April to September 2011. All the pandas chosen lived in captivity in the same base, but they were separated in individual zones by fences. The same food and water were provided daily for all these pandas by trained persons. Although these pandas lived separately for most of the time, they were sometimes pooled in the same zone for visitors during holiday seasons. Vaginal secretions were taken from mature female pandas (4 to 5 years of age) during a health check under anesthesia. Vaginal secretion samples were taken by cotton swab for *E. coli* isolation. Meanwhile, fecal pats from these selected pandas were also taken to isolate *E. coli*. Other fecal isolates were from pandas aged between 0 and 10 years living in the same base. The swabs and fecal samples were immediately transported on ice to the laboratory at Sichuan Agricultural University (Ya'an, Sichuan, China) and processed within <3 h. The swabs were broken off into tubes containing 5 ml of buffered peptone water (BPW; Beijing Land Bridge Technology Ltd., Beijing, China) and incubated at 37°C for 18 to 24 h. Fecal samples were diluted 1:10 in BPW and incubated at 37°C for 18 to 24 h. Following incubation, a loopful of the enrichment broth was streaked onto MacConkey agar (MAC; Beijing Land Bridge Technology Ltd.) plates and incubated at 37°C for 18 to 24 h. One or two putative *E. coli* isolates on MAC (bright pink with a dimple) per sample were transferred to eosin methylene blue agar (EMB; Beijing Land Bridge Technology Ltd.) plates for further purification and incubated at 37°C for 18 to 24 h. Suspect *E. coli* isolates on EMB (green colonies with a metallic sheen) were taken for biochemical tests. Indole-positive and oxidase-negative isolates were presumptively identified as *E. coli* and confirmed by PCR detection of the β -D-glucuronidase gene (*uidA* [*E. coli* specific]) (15). All isolates were stored in tryptic soy broth containing 15% glycerol at -80°C until use. All of the isolates were sent to the China Institute of Veterinary Drug Control, Beijing, China, to determine O antigens, using 166 O antisera.

ExPEC and diarrheagenic *E. coli* screening. ExPEC isolates were detected with a multiplex PCR for the following virulence-associated markers: *sfa/foc* (S and F1C fimbriae), *papA* and/or *papC* (P fimbriae), *iutA* (aerobactin receptor), *afa/dra* (Dr-antigen-binding adhesins), and *kpsMT II* (group 2 capsular polysaccharide units). ExPEC isolates were confirmed by the presence of at least two of the above-described five markers (16). DEC isolates were detected by multiplex PCR, as previously described (17), for the following virulence gene markers: *eae* for enteropathogenic *E. coli* (EPEC), *stx* for Shiga toxin-producing *E. coli* (STEC), *elt* and *est* for enterotoxigenic *E. coli* (ETEC), *ipaH* for enteroinvasive *E. coli* (EIEC), and *aggR* for enteroaggregative *E. coli* (EAEC).

Phylogenetic grouping. All of the *E. coli* isolates were assigned to one of the four phylogenetic groups (groups A, B1, B2, and D) by a multiplex PCR-based method as previously described (6), using three sets of primers (for *chuA*, *yjaA*, and the DNA fragment TspE4.C2).

Antimicrobial susceptibility testing. Antimicrobial susceptibility tests were performed by the agar dilution method for ampicillin (AMP) (resistance breakpoint, ≥ 32 μ g/ml), amoxicillin-clavulanate (AMC) (≥ 32 and 16 μ g/ml, respectively), chloramphenicol (CHL) (≥ 32 μ g/ml), nalidixic acid (NAL) (≥ 32 μ g/ml), ciprofloxacin (CIP) (≥ 4 μ g/ml), gentamicin (GEN) (≥ 8 μ g/ml), kanamycin (KAN) (≥ 25 μ g/ml), amikacin (AMK) (≥ 32 μ g/ml), cefoxitin (FOX) (≥ 32 μ g/ml), cefoperazone (CPZ) (≥ 64 μ g/ml), ceftriaxone (AXO) (≥ 64 μ g/ml), tetracycline (TET) (≥ 16 μ g/ml), and trimethoprim-sulfamethoxazole (SXT) (≥ 8 and 152 μ g/ml, respectively). Results were interpreted in accordance with Clinical and Laboratory Standards Institute criteria (18). *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were used as control strains.

PFGE. Pulsed-field gel electrophoresis (PFGE) using XbaI was performed to determine genomic DNA fingerprints of *E. coli* isolates as previously described (19). PFGE results were analyzed by using BioNumerics software (Applied-Maths, Kortrijk, Belgium), and banding patterns were compared by using Dice coefficients with a 1.5% band position tolerance. Genome DNA of *Salmonella enterica* serovar Branderup strain H9812 digested with XbaI was used as a molecular size marker. The Simpson index (*D*) was determined as previously described (20, 21), to assess the

TABLE 1 Serogroup distributions of 97 *E. coli* isolates from giant pandas

O type ^a	No. (%) of isolates of each serogroup from sample type		P value
	Fecal (<i>n</i> = 67)	Vaginal (<i>n</i> = 30)	
4	0	3 (10)	0.028
18	1 (1)	7 (23)	<0.001
88	5 (7)	6 (20)	0.072
158	3 (4)	0	0.325
167	6 (9)	2 (7)	0.705
Others	35 (52)	5 (17)	0.002
OR	5 (7)	5 (17)	0.309
NT	12 (18)	2 (7)	0.253

^a Others are serogroups detected in <3 isolates; OR, O-rough; NT, not serogroupable.

diversity of the *E. coli* populations. Simpson's *D* is an index ranging from 0 to 1, where higher values represent higher strain diversity.

Statistical analysis. Chi-square (χ^2) or Fisher's exact test was performed with SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA) for Windows, and a probability value of <5% was considered to be significant. Chi-square (χ^2) or Fisher's exact test was used to test the null hypothesis of equal prevalence rates of virulence genes, serotype, or antimicrobial resistance between fecal *E. coli* and vaginal *E. coli* isolates.

RESULTS

Serotyping. Of 97 isolates, 73 (75%) were typeable, including 5 isolates that reacted with two antisera. The remaining 24 isolates included 14 nontypeable and 10 rough isolates when using 166 antisera (see Fig. S1 in the supplemental material). Thirty-five different O serogroups were identified among 73 typeable *E. coli* isolates (see Fig. S1 in the supplemental material). The most prevalent serogroups in all *E. coli* isolates were O88 (11%; 11/97), O18 and O167 (each 8%; 8/97), and O4 and O158 (each 3%; 3/97) (Table 1). The percentage of isolates belonging to serogroups O4 and O18 was significantly higher ($P < 0.05$) for vaginal secretion samples than for fecal samples (Table 1). The remaining *E. coli* isolates were scattered among 30 other serogroups, with <3 isolates per serogroup (Table 1).

Thirty-one different O serogroups were identified among 50 typeable isolates from fecal samples. Serogroups O167 (12%; 6/50) and O88 (10%; 5/50) were most frequently identified in 50 typeable isolates from fecal samples (see Fig. S1 in the supplemental material). Nine different O serogroups were identified among 23 typeable isolates from vaginal secretions (see Fig. S1 in the supplemental material).

ExPEC and DEC. The 97 *E. coli* strains were screened by PCR for five ExPEC-defining virulence markers. *sfa/foc* (24%; 23/97) was most frequently detected, followed by *papC* and *kpsMT II* (23%; 22/97 each), *papA* (7%; 7/97), *iutA* (2%; 2/97), and *afa/dra* (none). The positive rates for *papA*, *papC*, and *sfa/foc* differed significantly ($P < 0.05$) between vaginal isolates and fecal isolates (Table 2). Nineteen (20%) *E. coli* isolates exhibited at least two of the five virulence markers and were considered ExPEC (Table 2). The percentage of ExPEC isolates was significantly higher ($P < 0.05$) among vaginal isolates (57%; 17/30) than among fecal isolates (3%; 2/67). All *E. coli* strains were also screened for four DEC-defining virulence markers. Two virulence markers, *aggR* and *ipaH* (each detected in two isolates), were detected in four isolates from fecal samples, while no DEC-defining virulence markers were detected in isolates from vaginal secretion samples.

TABLE 2 Prevalence of DEC, ExPEC, and ExPEC-defining virulence markers in *E. coli* isolated from giant pandas

Virulence factor	No. (%) of strains from indicated sample type		P value
	Fecal (n = 67)	Vaginal (n = 30)	
<i>ipaH</i>	2 (3)	0	0.855
<i>aggR</i>	2 (3)	0	0.855
Other DEC virulence markers	0	0	1.000
<i>papA</i>	0	7 (23)	<0.001
<i>papC</i>	5 (7)	17 (57)	<0.001
<i>sfa/foc</i>	7 (10)	16 (53)	<0.001
<i>afa/dra</i>	0	0	1.000
<i>iutA</i>	1 (1)	1 (3)	0.525
<i>kpsMT II</i>	12 (18)	10 (33)	0.094
ExPEC ^a	2 (3)	17 (57)	<0.001

^a Defined as isolates with at least two of the following virulence markers: *papA* and/or *papC*, *afa/dra*, *sfa/foc*, *iutA*, and *kpsMT II*.

Phylogenetic grouping. PCR analysis of the 97 isolates showed that 28/97 (29%) isolates belonged to phylogenetic group A, 56/97 (58%) belonged to group B1, 5/97 (5%) belonged to group B2, and 8/97 (7%) belonged to group D (Table 3). The percentage of isolates belonging to group B1 was significantly higher ($P < 0.05$) for fecal samples than for vaginal secretion samples, while the percentage of fecal isolates belonging to groups B2 and D was significantly lower ($P < 0.05$) than the percentage of vaginal isolates (Table 3).

Antimicrobial susceptibility testing. The 97 *E. coli* strains displayed resistance most frequently to trimethoprim-sulfamethoxazole (48%), followed by nalidixic acid (47%), tetracycline (25%), ampicillin (18%), kanamycin and ceftriaxone (15% each), gentamicin (13%), cefoxitin (10%), cefoperazone (9%), chloramphenicol and amikacin (7% each), ciprofloxacin (3%), and amoxicillin-clavulanic acid (2%). Percent resistances to chloramphenicol, cefoxitin, and ceftriaxone differed significantly ($P < 0.05$) between vaginal isolates and fecal isolates (Table 4). Seventy-four *E. coli* isolates (76%) were resistant to at least one antimicrobial, 35 (36%) were resistant to three or more, and 2 (2%) were resistant to nine (data not shown).

PFGE. All of the *E. coli* isolates were analyzed for genetic relatedness using PFGE with XbaI. Except for 5 isolates that were not typeable by using the enzyme chosen, the remaining 92 isolates were categorized into 74 PFGE patterns (see Fig. S1 in the supplemental material). The most predominant PFGE pattern observed was pattern 36 (P36) (5 isolates), followed by P60 (4 isolates) and

TABLE 3 Phylogenetic group distribution of 97 *E. coli* isolates from giant pandas

Phylogenetic group	No. (%) of isolates of each phylogenetic group from sample type		P value
	Fecal (n = 67)	Vaginal (n = 30)	
A	18 (27)	10 (33)	0.516
B1	48 (72)	8 (27)	<0.001
B2	1 (1)	4 (13)	0.031
D	0 (0)	8 (27)	<0.001

TABLE 4 Antibiotic resistances of 97 *E. coli* strains isolated from giant pandas

Antibiotic ^a	Breakpoint (μg/ml)	No. (%) of resistant isolates from indicated sample type		P value
		Fecal (n = 67)	Vaginal (n = 30)	
AMP	≥32	10 (15)	7 (23)	0.314
AMC	≥32–16	1 (1)	1 (3)	0.525
COT	≥8–152	35 (52)	12 (40)	0.265
CHL	≥32	2 (3)	5 (17)	0.028
NAL	≥32	29 (43)	17 (57)	0.222
CIP	≥4	1 (1)	2 (7)	0.225
GEN	≥16	10 (15)	3 (10)	0.749
KAN	≥64	13 (19)	2 (7)	0.137
AMI	≥32	7 (10)	0 (0)	0.096
FOX	≥32	10 (15)	0 (0)	0.029
CPZ	≥64	8 (12)	1 (3)	0.267
AXO	≥4	14 (21)	1 (3)	0.033
TET	≥16	15 (22)	9 (30)	0.422

^a All isolates were susceptible to ampicillin (AMP), amoxicillin-clavulanic acid (AMC), trimethoprim-sulfamethoxazole (COT), chloramphenicol (CHL), nalidixic acid (NAL), ciprofloxacin (CIP), gentamicin (GEN), kanamycin (KAN), amikacin (AMI), cefoxitin (FOX), cefoperazone (CPZ), ceftriaxone (AXO), tetracycline (TET), and erythromycin (ERY).

P59 (3 isolates). The other 8 groups of isolates sharing 100% homology belonged to P3, P9, P14, P15, P51, P52, P54, P55, and P56 (each with 2 isolates). Certain isolates with identical patterns, were recovered from different giant pandas. For example, isolates exhibiting P3 were recovered from pandas Guoguo and Haizi, and P60 isolates were recovered from pandas Juxiao, W, Jini, and Zhika. Isolates with identical P52 (fecal and vaginal) were recovered from different sources (see Fig. S1 in the supplemental material). The genetic diversity (*D*) value for fecal isolates was 0.989, and for vaginal isolates, it was 0.972.

DISCUSSION

One important cause of death of giant pandas is infection caused by pathogenic bacteria, especially *E. coli* (2, 3). Hemorrhagic enterocolitis, systemic sepsis, and deaths have been caused by *E. coli* infections of pandas (3, 9). However, reports of *E. coli* in giant pandas are relatively scarce. In this study, 97 *E. coli* isolates from giant pandas were analyzed for serogroups, phylogenetic backgrounds, antimicrobial resistances, PFGE profiles, and virulence factors indicative of pathogenicity.

Many serogroups of *E. coli* strains from pandas are associated with intestinal and/or extraintestinal infections. For example, *E. coli* O88 strains are associated with diarrhea (22, 23) and avian-pathogenic *E. coli* (APEC) infections (24). *E. coli* O18 strains caused neonatal meningitis and urinary tract infections in humans (25, 26) and APEC infections (11). *E. coli* O4 strains caused urinary tract infections in humans (27) and diarrhea in dogs (10). There is scarce information on the serogroups of *E. coli* strains causing intestinal and extraintestinal infections in pandas. Since the serogroups of panda *E. coli* isolates in this study overlapped those causing human and animal infections, their potential to cause infection in pandas necessitates attention and further exploration.

ExPEC strains were much more common in vaginal secretion

samples than in fecal samples. In contrast, diarrheagenic *E. coli* strains were detected only in fecal samples. The pathogenicity of these ExPEC isolates needs further investigation. The presence of EAEC and EIEC strains in fecal samples is also of concern, since these fecal materials could serve as sources of enteric infection of other pandas living in the same area.

In general, most virulent extraintestinal *E. coli* strains belong to group B2 or D (28), whereas commensal strains (29) and strains derived from veterinary species (12) belong mostly to group A or B1. Our analysis of panda isolates showed that only a small fraction of isolates from fecal sources belonged to groups B2 and D, while 40% of vaginal isolates fell into these two phylogroups, which was in agreement with a previous study showing that 40% of porcine ExPEC strains belonged to these two virulent groups (12).

Compared with antimicrobial resistance rates of *E. coli* isolates from other animals (13) and from the environment (30) in China, the rate of resistance to antimicrobials in isolates from giant pandas was much lower. For antibiotic resistance of *E. coli* isolates from pandas, Zhang et al. (31) reported that 32% (19/59) of the fecal bacteria from pandas were resistant to at least one antimicrobial and that 17% (10/59) were resistant to three or more antimicrobials, rates which were lower than the rates found in this study (76% and 36%, respectively). Compared to another report of antimicrobial resistances of 38 fecal *E. coli* strains isolated from pandas living in the same area in 2008 (32), the percent resistances to sulfamethoxazole-trimethoprim (from 0% to 48%) and gentamicin (from 3% to 13%) increased. Since sulfamethoxazole-trimethoprim and gentamicin have not been used as therapeutic drugs in pandas, the increased resistance may be explained by the possibility that these pandas can acquire antimicrobial-resistant bacteria through contact with humans and domestic animals or in the environment (33). Penicillins and fluoroquinolones have been frequently used as therapeutic drugs in pandas, which may account for the high resistance rate detected. Therefore, it is important to keep imprudent use of antimicrobials in mind. Drug combination or drug rotation combined with routine surveillance of antimicrobial resistance in panda *E. coli* isolates are suggested to prevent antimicrobial resistance and help select drugs for treating *E. coli* infections in pandas.

Simpson's index of diversity based on PFGE patterns indicated great diversity in both vaginal and fecal isolates. Certain *E. coli* isolates with identical PFGE patterns were recovered from different giant pandas. This may reflect a clonal spread of specific strains among different pandas. In contrast, common PFGE patterns were rarely shared (except for isolates of P52) in *E. coli* strains isolated from fecal and vaginal secretion samples. This may suggest that *E. coli* isolates from fecal samples are generally not associated with those from vaginal samples.

In summary, our study revealed that many characteristics of *E. coli* isolates from giant pandas, including serogroups, phylogenetic groups, and virulence profiles, overlapped those of isolates causing human or animal infections. In addition, these *E. coli* isolates exhibited antimicrobial resistance and were genetically diverse. Attention should be paid to the presence of these potentially pathogenic *E. coli* isolates in giant pandas, and further research to explore their role in causing infections in pandas is warranted.

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