Serotypes, virulence factors and antimicrobial susceptibility of vaginal and fecal isolates of *Escherichia coli* from the giant panda

Xin Wang¹,², Qigui Yan³*, Xiaodong Xia¹, Yanming Zhang²*, Desheng Li⁴, Chengdong Wang⁴, Shijie Chen⁵, Rong Hou⁶

¹ College of Food Science and Engineering, Northwest A & F University, Yangling, Shaanxi 712100, China
² College of Veterinary Medicine, Northwest A&F University, Yangling, Shaanxi 712100, China
³ College of Veterinary Medicine, Sichuan Agricultural University, Ya'an Sichuan 625014, China
⁴ China Conservation and Research Center for the Giant Panda, Ya'an, Sichuan 625000, China
⁵ Sichuan Entry-Exit Inspection and Quarantine Bureau, Chengdu, Sichuan 610041, China
⁶ Chengdu research Base of Giant Panda Breeding, Chengdu, Sichuan 610081, China

※Corresponding authors:
Qigui Yan,
College of Veterinary Medicine, Sichuan Agricultural University
46 Xinkang Road, Yu Cheng District, Ya’an, Sichuan, 625014 China
Email: yanqigui@126.com
Tel:+86+835-2885951; Fax:+86+835-2885302.

or Yanming Zhang,
Escherichia coli isolates from giant panda

Keywords: Escherichia coli; Giant panda; Serotypes; Virulence genes; Antimicrobial resistance; PFGE.
Abstract

Although *Escherichia coli* (*E. coli*) typically colonize the intestinal tract and vagina of the giant pandas, it has caused enteric and systemic disease in the giant pandas and greatly impact 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 group, antimicrobial susceptibility, and 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 in all *E. coli* isolates were O88, O18, O167, O4 and O158. ExPEC mostly were detected in vaginal isolates and DEC only detected in fecal isolates. Phylogenetic group B1 predominated in fecal isolates, while groups B2 and D were frequently detected in vaginal isolates. Resistance was most frequently observed to trimethoprim/sulfamethoxazole, followed by nalidixic acid, tetracycline. All except five isolates were typeable 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* 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 in the giant panda.

**Keywords:** *Escherichia coli*, Giant panda; Serotypes; Virulence genes; Antimicrobial resistance; PFGE.
Introduction

The giant panda or panda (*Ailuropoda melanoleuca*) is one of the most endangered and rare animals in the world. Today, it only lives in Sichuan, Shaanxi and Gansu provinces in China (1). The leading cause of death of panda is due to various diseases, among 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*, *Arizona* spp., *Pseudomonas aeruginosa*, *Yersinia enterocolitica* and *Clostridium welchii*. Those entric disorders seriously affect the digestion and absorption of food, compromise the immune system, and even cause serious implications and death, which endangers the survival of the giant panda (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, diarrheagenic *E. coli* (DEC, also called intestinal pathogenic *E. coli*) and extraintestinal pathogenic strains (4). Intestinal pathogenic *E. coli* strains typically elicit diarrheal symptoms, while extraintestinal pathogenic *E. coli* (ExPEC) strains cause urinary tract infections (UTIs), sepsis, abdominal infections, meningitis, cellulitis, osteomyelitis, and wound infections (5).

Based on the phylogenetic backgrounds, the *E. coli* population could be classified into 4 major phylogroups (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 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 the giant pandas, although several *E. coli* clones could 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* from various animal species have been investigated extensively (10-14). However, little is known about the distribution and characteristics of *E. coli* in the giant panda. Therefore, we carried out this study to determined serogroup, phylogenetic group, antimicrobial susceptibility, and PFGE profiles of 97 *E. coli* strains from 21 giant pandas. Moreover, the presence of virulence genes used to define pathogroups 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 secretions, were collected from 21 *healthy* female giant pandas
living in Chinese Giant Panda Pressing Base of Bi Feng Xia (Sichuan province in China) in two periods: from 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 those pandas by trained persons. Although those pandas lived separately for most of the time, they were sometimes pooled together in the same zone for visitors during holiday seasons. Vaginal secretions were taken from mature female pandas (4-5 years of age) during health check under anesthesia. Vaginal secretions 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 0-10 years of age 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 less than 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 out on 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 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 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 were confirmed by the presence of at least two of the above five markers (16). Diarrheagenic *E. coli* (DEC) were detected with a 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), and *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 (A, B1, B2, and D) by a multiplex PCR-based method as previously described (6) using three sets of primers (*chuA*, *yjaA* and DNA fragment *TspE4.C2***).

**Antimicrobial susceptibility testing**

Antimicrobial susceptibility tests were performed by the agar dilution method for Ampicillin (AMP) (resistance breakpoint ≥32 μg/ml), Amoxicillin/clavulanate (AMC)
(≥32/16μg/ml), Chloramphenicol (CHL) (≥32μg/ml), Nalidixic acid (NAL) (≥32μg/ml), Ciprofloxacin (CIP) (≥4μg/ml), Gentamicin (GEN) (≥8μg/ml), Kanamycin (KAN) (≥25μg/ml), Amikacin (AMK) (≥32μg/ml), Cefoxitin (FOX) (≥32μg/ml), Cefoperazone (CPZ) (≥64μg/ml), Ceftriaxone (AXO) (≥64μg/ml), Tetracycline (TET) (≥16μg/ml), and Trimethoprim/sulfamethoxazole (SXT) (≥8/152μg/ml). Results were interpreted in accordance with the Clinical Laboratory Standards Institute criteria (18).

Escherichia coli ATCC 25922 and S. aureus ATCC 29213 were used as control strains.

Pulsed-field gel electrophoresis (PFGE)

PFGE using XbaI was performed to determine genomic DNA fingerprint of E. coli isolates as previously described (19). PFGE results were analyzed by the 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 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 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

The Chi-square (χ²) or Fisher’s exact tests were performed with SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA) for Windows and a probability value of less than 5% was considered to be significant. The Chi-square (χ²) or Fisher's exact tests were 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 (Fig. S). Thirty-five different O serogroups were identified among 73 typeable *E. coli* isolates (Fig. S). 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 serogroup O4 and O18 was significantly higher (*P* < 0.05) from vaginal secretion than from fecal samples (Table 1). The remaining *E. coli* isolates were scattered among 30 other serogroups, with fewer than 3 isolates per serogroup (Table 1).

Thirty-one different O serogroups were identified among 50 typeable isolates from fecal samples. Serogroup O167 (12%, 6/50) and O88 (10%, 5/50) were most frequently identified in 50 typeable isolates from fecal samples (Fig. S). Nine different O serogroups were identified among 23 typeable isolates from vaginal secretions (Fig. S).

ExPEC and DEC

The 97 *E. coli* strains were screened by PCR for five ExPEC-defining virulence markers. *Sfa/foc* (24%, 23/97) were 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 rate of *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 as 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 secretions.

**Phylogenetic grouping**

PCR analysis of the 97 isolates showed that 28/97 (29%) isolates belonged to phylogenetic group A, 56/97 (58%) to B1, 5/97 (5%) to B2 and 8/97 (7%) to D (Table 3). The percentage of isolates belonging to group B1 was significantly higher ($P < 0.05$) from fecal samples than from vaginal secretion, while the percentage of fecal isolates belonging to group B2 and D was significantly lower ($P < 0.05$) than 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% for each), ciprofloxacin (3%) and amoxicillin/clavulanic acid (2%). Percent resistance 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%) to three or more, and 2 (2%) 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 which were not typeable using the enzyme chosen, the remaining 92 isolates were categorized into 74 PFGE patterns (P) (Fig. S). The most predominant PFGE patterns were observed in P36 (5 isolates), followed by P60 (4 isolates) and P59 (3 isolates). Other 8 groups of isolates sharing 100% homology were P3, P9, P14, P15, P51, P52, P54, P55 and P56 (each 2 isolates). Isolates with identical P3 (Guoguo and Haizi), P9 (Mianzhu and Qianqian), P14 (W and Guoguo), P15 (Guoguo and Huanhua) P36 (Mianzhu and Qianqian), P54 (Jini and Xixi), P55 (L2 and Y1), P59 (Zhika and Yingmei) and P60 (Juxiao, W, Jini and Zhika) were recovered from different giant pandas. Isolates with identical P52 (fecal and vaginal) recovered from different sources (Fig. S). The genetic diversity (D) for fecal isolates was 0.989, and for vaginal isolates it was 0.972.
Discussion

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

Many serogroups of *E. coli* 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 human (27) and diarrhea in dog (28). There is scarce information on the serogroup of *E. coli* causing intestinal and extraintestinal infections in panda. Since the serogroups of panda *E. coli* isolates in this study overlapped with those causing human and animal infections, their potential to cause infection in panda necessitate attention and further exploration.

ExPEC were much more common in isolates from vaginal secretions than in isolates from fecal. In contrast, diarrheagenic *E. coli* were only detected in isolates from fecal samples. The pathogenicity of those ExPEC needs further investigation. The presence of EAEC and EIEC found from fecal samples is also of concern since these fecal materials could serve as sources of enteric infection for other pandas.
In general, most virulent extraintestinal *E. coli* strains belong to group B2 or D (29), whereas commensal strains (30) and strains derived from veterinary species (12) mostly belong to groups A or B1. Our analysis of panda isolates showed that only a small fraction of isolates from fecal source 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 belonged to these two virulent groups (12).

Compared with antimicrobial resistance rates of *E. coli* isolates from other animals (13) and from environment (31) in China, resistance to antimicrobials in isolates from giant pandas was much lower. For antibiotic resistance of *E. coli* in panda, Zhang et al. (32) have reported that 32% (19/59) of the fecal bacteria from panda were resistant to at least one antimicrobial, and 17% (10/59) were resistant to three or more antimicrobials, which were lower than the rates found in this study (76% and 36%, respectively). Compared to another report studying antimicrobial resistance of 38 fecal *E. coli* isolated from pandas living in the same area in 2008 (33), percent resistance to sulfamethoxazole/trimethoprim (from 0% to 48%) and gentamicin increased (from 3% to 13%). Since sulfamethoxazole/trimethoprim and gentamicin have not been used as therapeutic drugs of pandas, the increase of resistance may be explained by that these pandas can acquire antimicrobial-resistant bacteria through contact with humans and domestic animals or through the environment (34). Penicillins and fluoroquinolones have been frequently used as
therapeutic drugs of 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, are suggested to prevent antimicrobial resistance and help select drugs for treating E. coli infections in panda.

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 in 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 secretions.

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

Acknowledgement

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Reference


Table 1  Serogroups distribution of 97 *E. coli* isolates from giant panda

<table>
<thead>
<tr>
<th>O type</th>
<th>Prevalence of each serogroup, no. (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fecal isolate (n=67)</td>
<td>Vaginal isolates (n=30)</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>3(10%)</td>
</tr>
<tr>
<td>18</td>
<td>1(1%)</td>
<td>7(23%)</td>
</tr>
<tr>
<td>88</td>
<td>5(7%)</td>
<td>6(20%)</td>
</tr>
<tr>
<td>158</td>
<td>3(4%)</td>
<td>0</td>
</tr>
<tr>
<td>167</td>
<td>6(9%)</td>
<td>2(7%)</td>
</tr>
<tr>
<td>Othersa</td>
<td>35(52%)</td>
<td>5(17%)</td>
</tr>
<tr>
<td>OR</td>
<td>5(7%)</td>
<td>5(17%)</td>
</tr>
<tr>
<td>NT</td>
<td>12(18%)</td>
<td>2(7%)</td>
</tr>
</tbody>
</table>

*Others: serogroups detected in less than 3 isolates; OR: O-rough; NT: not serogroupable*
Table 2 Prevalence of DEC, ExPEC and ExPEC-defining virulence markers in *E. coli* isolated from giant panda

| Virulence factors/ExPEC<sup>a</sup> | No. (%) of strains from giant panda |  
| --- | --- | --- | --- |
|  | Fecal (n=67) | Vaginal secretion (n=30) |  
| *ipaH* | 2(3%) | 0 | 0.855 |
| *aggR* | 2(3%) | 0 | 0.855 |
| Other DEC virulence markers | 0 | 0 | 1.000 |
| *papA* | 0 | 7(23%) | < 0.001 |
| *papC* | 5(7%) | 17(57%) | < 0.001 |
| *sfa/foc* | 7(10%) | 16(53%) | < 0.001 |
| *afa/dra* | 0 | 0 | 1.000 |
| *iutA* | 1(1%) | 1(3%) | 0.525 |
| *kpsMT II* | 12(18%) | 10(33%) | 0.094 |
| ExPEC<sup>a</sup> | 2(3%) | 17(57%) | < 0.001 |

DEC: diarrheagenic *E. coli*; ExPEC: extraintestinal pathogenic *E. coli*

<sup>a</sup>Defined as isolates with ≥ two of the following five virulence markers: *papA* and/or *papC*, *afa/dra*, *sfa/foc*, *iutA*, *kpsMT II*. 
Table 3 Phylogenetic group distribution of 97 E. coli isolates from giant panda

<table>
<thead>
<tr>
<th>Phylogenetic groups</th>
<th>Prevalence of each phylogenetic group, no. (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fecal isolates (n=67)</td>
<td>Vaginal isolates (n=30)</td>
</tr>
<tr>
<td>A</td>
<td>18 (27%)</td>
<td>10 (33%)</td>
</tr>
<tr>
<td>B1</td>
<td>48 (72%)</td>
<td>8 (27%)</td>
</tr>
<tr>
<td>B2</td>
<td>1 (1%)</td>
<td>4 (13%)</td>
</tr>
<tr>
<td>D</td>
<td>0 (0%)</td>
<td>8 (27%)</td>
</tr>
</tbody>
</table>
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), erythromycin (ERY).