Antimicrobial resistance of *Escherichia coli*, *Enterobacter* spp., *Klebsiella pneumoniae* and *Enterococcus* spp. isolated from the feces of giant panda

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**Abstract**

*Background:* *Escherichia coli*, *Enterobacter* spp., *Klebsiella pneumoniae* and *Enterococcus* spp., common gut bacteria in giant pandas, include opportunistic pathogens. The giant panda is an endangered species, classified as vulnerable by the World Wildlife Foundation. Continuous monitoring for the emergence of antimicrobial resistance (AMR) among bacterial isolates from giant pandas is vital not only for their protection but also for public health.

*Results:* A total of 166 *E. coli*, 68 *Enterobacter* spp., 116 *K. pneumoniae* and 117 *Enterococcus* spp. isolates were collected from fecal samples of 166 giant pandas. In the antimicrobial susceptibility tests, 144 *E. coli* isolates, 66 *Enterobacter* spp. isolates, 110 *K. pneumoniae* isolates and 43 *Enterococcus* spp. isolates were resistant to at least one antimicrobial. The resistant isolates carried antimicrobial resistance genes (ARGs), including *sul3*, *bla*TEM, *bla*SHV and *tetA*. The differences in the prevalence of the *bla* types implied that the genetic basis for β-lactam resistance among the *E. coli*, *Enterobacter* spp. and *K. pneumoniae* isolates was different. The strain *K. pneumoniae* K85 that was resistant to sixteen antimicrobials was selected for whole genome sequencing. The genome contained Col440I, IncFIBK and IncFIIK plasmids and altogether 258 ARGs were predicted in the genome; 179 of the predicted ARGs were efflux pump genes. The genetic environment of the β-lactamase genes *bla*CTX-M-3 and *bla*TEM-1 in the *K. pneumoniae* K85 genome was relatively similar to those in other sequenced *K. pneumoniae* genomes. In comparing the giant panda age groups, the differences in the resistance rates among *E. coli*, *K. pneumoniae* and *Enterobacter* spp. isolates suggested that the infections in giant pandas of different age should be treated differently.

*Conclusions:* Antimicrobial resistance was prevalent in the bacterial isolates from the giant pandas, implying that the gut bacteria may pose serious health risks for captive giant pandas. The resistance genes in the genome of *K. pneumoniae* K85 were associated with insertion sequences and integron-integrase genes, implying a potential for the further spread of the antimicrobial resistance.

**Keywords:** Antimicrobial resistance, Resistance genes, Whole-genome sequencing, Giant panda

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approximately 600 by the end of 2019. Although the number of both wild and captive pandas has increased, the giant pandas are still endangered due to several threats. Intestinal tract diseases caused by pathogenic bacteria has become a considerable threat to the health of giant pandas [2]. Escherichia coli, Enterobacter spp., Klebsiella and Enterococcus spp. are common gut bacteria in humans and other animals, including giant pandas [3–5]. These species play important commensal roles in gut; however, they are also opportunistic pathogens, and can cause various diseases [6–8]. For example, some E. coli strains cause hemorrhagic colitis, and these enterohaemorrhagic E. coli have been isolated from giant pandas [9]. Enterobacter spp., K. pneumoniae and Enterococcus faecium have been associated with hospital-acquired infections and outbreaks [10–15]. Clinical infections caused by Enterococcus spp. have been increasing in recent years [16], and Klebsiella and Enterobacter spp. can cause a wide range of infections [12, 17–19].

Antimicrobials have been widely used to prevent and cure infectious diseases in captive giant pandas in recent decades [20–22]. However, with the widespread use of antimicrobials, the number of drug-resistant strains has increased and the development and spread of multidrug-resistant (MDR) bacteria in humans and the environment has accelerated [23]. In China, more antimicrobial agents are consumed than in most other countries. According to a 2007 survey, almost half of the 210,000 tons of antimicrobials produced in China were used in livestock as therapeutic drugs and feed additives [24]. In addition, antimicrobials like ceftriaxone sodium are used not only in humans but also in giant pandas [25]. Thus, antimicrobial resistant strains may develop in giant pandas and spread to humans and other animals.

Antimicrobial resistance has caused serious problems in clinical practice [26]. Infections by Klebsiella spp., especially K. pneumoniae, are frequently caused by MDR strains that produce extended-spectrum β-lactamases (ESBLs; mainly including blaTEM, blaCTX,M, blashv and blagen types) [19, 26]. K. pneumoniae may be also naturally resistant to certain antimicrobials, including ampicillin, amoxicillin, carbenicillin and ticarcillin [27, 28]. Likewise, Enterobacter spp., especially Enterobacter cloacae, may be naturally resistant to, for example, ampicillin, kanamycin and tetracycline [7]. Generally, Enterococcus spp. are intrinsically resistant to many antimicrobials and can easily acquire resistance to other agents [29]. Acquired high-level aminoglycoside or penicillin resistance, as well as erythromycin or tetracycline resistance, have increased among Enterococcus spp. [16, 30, 31].

Several investigations have been carried out to monitor the distribution of antimicrobials and disinfectant resistance genes in E. coli and K. pneumoniae isolates from the giant pandas [2, 20, 22, 32]. In giant pandas, E. coli infections were frequently caused by MDR strains [2, 20, 32]. To our knowledge, detailed gene and genome level information on the antimicrobial resistant bacteria, especially on Enterobacter and Enterococcus spp., from giant pandas is still lacking. Therefore, comprehensive investigation at molecular level to monitor the distribution of antimicrobial resistant, opportunistic pathogens from giant pandas was needed. We isolated E. coli, Enterobacter spp., K. pneumoniae and Enterococcus spp. from giant panda feces and assessed their antimicrobial resistance and related genetic properties, with the aims to 1) characterize the antimicrobial resistance phenotypes and genotypes, 2) compare the antimicrobial resistance between the four taxa, and to 3) further understand the resistance based on whole-genome sequencing of a MDR K. pneumoniae isolate.

## Results

### Antimicrobial susceptibility of all isolates

A total of 166 E. coli, 68 Enterobacter spp., 116 K. pneumoniae and 117 Enterococcus spp. isolates were purified from fecal samples of 166 giant pandas. Only one isolate per genus per giant panda was kept for further analyses. In the antimicrobial susceptibility tests, 87% (n = 144) E. coli isolates, 97% (n = 68) Enterobacter spp. isolates, 95% (n = 110) K. pneumoniae isolates and 37% (n = 37) Enterococcus spp. isolates were resistant to at least one antimicrobial (Fig. 1, Supplementary Table S1).

Many of the isolates were resistant to at least three different antimicrobial classes and were considered MDR strains. Out of the E. coli isolates, 72% were resistant to sulfadiazine (SD), 38% to tetracycline (TET), and approximately 23% to amoxicillin (AML) and ampicillin (AMP) (Fig. 2); 18% (n = 29) were resistant to three or more tested antimicrobials (Supplementary Table S1). Out of the Enterobacter spp. isolates, 88% were resistant to AML, 84% to AMP, and 7% (n = 5) were MDR strains. Out of the K. pneumoniae isolates, 85% were resistant to AML, 73% to AMP, 47% to SD, and 15% (n = 17) were MDR strains. Out of the Enterococcus spp. isolates, 35% were resistant to TET, 29% to erythromycin (ERY), and 3% (n = 15) were MDR strains.

The prevalence of gentamicin (GEN) resistance was highest among the Enterobacter spp. isolates, and that of SD resistance was highest among the E. coli isolates and second highest among the K. pneumoniae isolates. The prevalence of AMP and AML resistances were highest and that of TET lowest among the Enterobacter spp. and K. pneumoniae isolates.
Antimicrobial resistant strains by giant panda sex and age

Antimicrobial resistant isolates were detected in 161 of the 166 giant pandas (Fig. 1). The difference in the proportion of antimicrobial resistant isolates from female and male giant pandas was limited to Enterococcus isolates: 26.9 and 46.0% of the isolates from females and males, respectively, were resistant to tetracycline ($P < 0.05$) (Fig. S1).

Among the E. coli isolates, the prevalence of resistance to six antimicrobials was highest in isolates from pandas in their infancy ($P < 0.05$) (Fig. 3a). All the E. coli isolates from infant and old pandas were resistant to SD, and the prevalence of SD resistance was lowest among isolates from adolescent pandas ($P < 0.05$). The prevalence of AMP resistance was higher among Enterobacter spp. isolates from adult pandas than among those from

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**Fig. 1** Antimicrobial resistance of E. coli, Enterobacter spp., K. pneumoniae and Enterococcus spp. isolates against 18 antimicrobials. The indicator on the right denotes the relationship between the antimicrobial resistance and color range. KAN, kanamycin; GEN, gentamicin; AZM, azithromycin; ERY, erythromycin; NOR, norfloxacin; OFX, ofloxacin; CIP, ciprofloxacin; LOM, lomefloxacin; LEV, levofloxacin; SD, sulfadiazine; TMP, trimethoprim; CRO, ceftriaxone; CFX, cefixime; AMP, ampicillin; AML, amoxicillin; ATM, aztreonam; IPM, imipenem; TET, tetracycline.

**Fig. 2** Antimicrobial resistance of E. coli, Enterobacter spp., K. pneumoniae and Enterococcus spp. isolates against 18 antimicrobial agents. KAN, kanamycin; GEN, gentamicin; AZM, azithromycin; ERY, erythromycin; NOR, norfloxacin; OFX, ofloxacin; CIP, ciprofloxacin; LOM, lomefloxacin; LEV, levofloxacin; SD, sulfadiazine; TMP, trimethoprim; CRO, ceftriaxone; CFX, cefixime; AMP, ampicillin; AML, amoxicillin; ATM, aztreonam; IPM, imipenem; TET, tetracycline. Different letters above columns indicate statistically significant differences at $P < 0.05$. 

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infant and adolescent pandas ($P < 0.05$) (Fig. 3b). For the *K. pneumoniae* isolates, the prevalence of resistance to four antimicrobials was highest in isolates from old pandas ($P < 0.05$), and the prevalence of SD resistance was highest in isolates from adult pandas ($P < 0.05$) (Fig. 3c). For *Enterococcus* spp. isolates, there was almost no significant difference in resistance to tested antimicrobials among giant pandas of different ages (Fig. 3d).

**Prevalence of ARGs**

The genotypes of antimicrobial resistant *E. coli*, *Enterobacter* spp., *K. pneumoniae* and *Enterococcus* spp. isolates were characterized by analyzing the ARGs in the isolates with different antimicrobial resistance phenotypes.

Among the *E. coli* isolates, *tetA* was detected in 67% (42/63) of the tetracycline-resistant isolates, *bla*<sub>TEM</sub> and *bla*<sub>CTX</sub> were detected in 36% (16/45) and 18% (8/45) of the β-lactam-resistant isolates, respectively, *sul2* and *sul3* were detected in 7% and 9% of the sulfonamide-resistant isolates, respectively, *qnrB* was detected in one of the eight fluoroquinolone-resistant isolates, and both *acc (3″)-IIa* and *ant (3″)-Ia* were detected in one of the five aminoglycoside-resistant isolates (Table 1).

The gene *tetA* was detected in 77% (10/13) of the tetracycline-resistant *Enterobacter* spp. isolates, *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub> and *bla*<sub>CTX</sub> were detected in 11% or less of the β-lactam-resistant isolates, *sul1* was detected in two of the eight sulfonamide-resistant isolates, and *ant (3″)-Ia* was detected in one of the nine aminoglycoside-resistant isolates (Table 1).

ARGs for β-lactam-resistance were detected in all the resistant *K. pneumoniae* isolates, with *bla*<sub>SHV</sub> in 83% (82/99) of them, *tetA* was detected in 76% (10/13) of the tetracycline-resistant isolates, and *sul1*, *sul2* and *sul3* were detected in 13% or less of the sulfonamide-resistant isolates. The only *K. pneumoniae* aminoglycoside-resistant isolate carried *acc (6′)-Ib* gene.

The gene *ermE* was detected in 24% of the macrolide resistant *Enterococcus* spp. isolates, and 35% of the tetracycline-resistant isolates carried *tetM* or *tetL* genes.

**Antibiotic resistance features in the *K. pneumoniae* K85 genome**

The strain *K. pneumoniae* K85 that was resistant to sixteen antimicrobials was selected for whole genome sequencing. The 1454 reads (Clean Data) were assembled into 91 contigs with a combined length of 5,514,535 bp. The longest contig was 368,946 bp. A total of 5349 ORFs were detected in the *K. pneumoniae* K85 genome with an average gene length of 897 bp. *K. pneumoniae* K85
Table 1 Resistance genes and genetic elements in antimicrobial resistant *E. coli*, *Enterobacter* spp., *K. pneumoniae* and *Enterococcus* spp. isolates from the feces of giant pandas in China

| Resistance phenotype | Resistance gene | Number of resistance genes or genetic elements / Number of antimicrobial-resistant isolates |
|----------------------|-----------------|------------------------------------------------------------------------------------------|
|                      | E. coli | Enterobacter | K. pneumoniae | Enterococcus |
| Aminoglycosides      | acc (3')-Ila | 1/5 | 0/9 | 0/1 | – |
|                      | aph (3')-Ila | 0/5 | 0/9 | 0/1 | – |
|                      | acc (6')-Ib | 0/5 | 0/9 | 1/1 | – |
|                      | ant (3')-Ia | 1/5 | 1/9 | 0/1 | – |
| Macrolides           | ermE | – | – | – | 8/34 |
| Quinolones           | qnrA | 0/8 | – | 0/3 | – |
|                      | qnrB | 1/8 | – | 1/3 | – |
| Sulfoximides         | sul1 | 1/121 | 0/8 | 7/54 | – |
|                      | sul2 | 9/121 | 2/8 | 4/54 | – |
|                      | sul3 | 11/121 | 0/8 | 3/54 | – |
| β-Lactams            | blaTEM | 16/45 | 4/62 | 7/99 | – |
|                      | blaOXA | 0/45 | 0/62 | 2/99 | – |
|                      | blaSHV-1 | 1/45 | 7/62 | 82/99 | – |
|                      | blaCTX | 8/45 | 5/62 | 8/99 | – |
| Tetracyclines        | tetA | 42/63 | 10/13 | 16/21 | – |
|                      | tetB | 2/63 | 1/13 | 0/21 | – |
|                      | tetC | 2/63 | 0/13 | 2/21 | – |
|                      | tetM | – | – | – | 8/23 |
|                      | tetL | – | – | – | 8/23 |

*, resistance gene not detected*

Table 2 Antimicrobial resistance genes in the genome of *K. pneumoniae* K85

| Mechanism of antibiotic resistance | Resistance genes | Number of genes |
|-----------------------------------|------------------|-----------------|
| Antibiotic efflux                 | aadA16, aph (3')-Ia, acc (6')-Ib-cr, blaCTX, blaTEM-1, ndmA, fosA5, mphA, mrr, cmv, arr-3, rhpB | 19 |
| Antibiotic inactivation           | tetA, baeR, kdpE, adefE, Escherichia coli CpxR, facT, emrB, cpxA, leuO, bcrA-1, bcrA, emrD, RpoH, hvmR, hpl1,181, lmrB, IhaA, macA, macB, mtdA, mtdG, mdtH, mdtK, mdtL, mdtM, DcmO, mcmO, mcmP, mepA, mepC, msbA, mvaB, norB, oleC, parA, parB, rpsA, rscB, salA, savB66, taeA, tcmA, tetA48, tetA48(60), tetB60, tetC, vgbA, yaiJ, Enterobacter cloacae rhaB, Escherichia coli rhaB, marA, ramA, aceE, adeE, adeF, adeS, baeA, cmeB, cmeC, cmeD, cmeF, emmA, emmK, emmR, emmY, Escherichia coli acrA, egkA, evg5, H-NS, mdtA, mdtF, mexA, mexK, mxxB, qnrM, qnrZ, qoxA, sideA, smeC, tcr3 | 179 |
| Antibiotic inactivation, antibiotic target alteration | tet34 | 1 |
| Antibiotic target alteration      | vanRF, vanRE, vanRM, vanG, vanHB, gypR, parY, EF-Tu, gymA, LhpT, murA, kasA, katG, ndhI, ileS, cysA, acr5, cssR, cibB, rimA (III), arrA, eptB, pmrE, pmrF, bacA | 30 |
| Antibiotic target alteration, antibiotic efflux | basR, basS | 2 |
| Antibiotic target protection      | mfd, qnrB2, qnrS1, tetT, vanR1, vanH5, vanT1, vanT4, vanTn, pmrC | 12 |
| Antibiotic target replacement     | mecC, dfrA3, dfrA5, dfrE, sul1, sul1 | 8 |
| Reduced permeability to antibiotic, resistance by absence | E. coli LamB | 2 |
| Reduced permeability to antibiotic | K. pneumoniae OmpK37 | 5 |

contained Col440l, IncFIBK, and IncFIIK plasmids. Altogether 258 ARGs were predicted in the *K. pneumoniae* K85 genome (Table 2). Altogether 179 of the predicted ARGs were efflux pump genes, and the rest were related to enzymatic inactivation of antimicrobials, alteration, protection and replacement of the antimicrobial target,
and reduced permeability to antimicrobials. The predicted aminoglycoside-modifying enzyme genes included aadA16, aph(3′)-Ia, and acc(6′)-Ib-cr that can simultaneously confer fluoroquinolone resistance. The predicted bla\textsubscript{CTX-M-3}, bla\textsubscript{SHV-93} and bla\textsubscript{TEM-1} confer resistance to β-lactams. In addition, genes encoding general mechanisms that mediate antibiotic resistance to fluoroquinolone (qnrB2 and qnrS1), sulfonamide (sul1 and sul3) and tetracycline (tet34 and tetT) were also predicted.

The genetic environment of the β-lactamase genes bla\textsubscript{CTX-M-3} and bla\textsubscript{TEM-1} in the K. pneumoniae K85 genome was relatively similar to those in other sequenced K. pneumoniae genomes (Fig. 4). The gene bla\textsubscript{TEM-1} was adjacent to bla\textsubscript{CTX-M-3} and this resistance region also included another two ARGs (floR and tetA) conferring resistance to tetracycline and florfenicol. More importantly, these antimicrobial resistance regions were flanked by various IS elements. The gene bla\textsubscript{TEM-1} was adjacent to bla\textsubscript{CTX-M-3} and this region also included floR and tetA that confer resistance to florfenicol and tetracycline, respectively. The isolate K. pneumoniae K85 harbored a class 1 integron gene cassette with resistance genes aac (6′)-Ib-cr, arr-3, dfrA5 and aadA16 (Fig. 5).

**Discussion**

We studied the distribution of antimicrobial resistant, opportunistic pathogens in giant panda guts by isolating E. coli, Enterobacter spp., K. pneumoniae and Enterococcus spp. from giant panda feces. The results showed that antimicrobial resistance was common among the isolates, ranging from 95% or more among the Enterobacter spp. and K. pneumoniae isolates to 37% among the Enterococcus spp. isolates.

Our results showed that five E. coli isolates were resistant to ten or more antimicrobials, implying that MDR E. coli may pose serious health risks for captive giant pandas. Compared to the 88 E. coli strains from giant pandas in Bifengxia, China [20], in our study the antimicrobial resistance range of the isolates was wider and the prevalence of resistance to amoxicillin was higher. However, the prevalence of resistances to six antimicrobials were lower than an earlier study on giant pandas from Wolong.
and Dujiangyan, the China Conservation and Research Center for Giant Panda [22], possibly partly due to the controlled use of antimicrobials [32]. In addition, the variation in antimicrobial resistance profiles at different times and sites may result from giant pandas obtaining antimicrobial-resistant bacteria via contacts with feeders, feeding environment or tourists that violate the feeding regulations of the zoos [32–34], thus increasing the risks of cross-infection and exposure to pathogens and ARGs through the digestive tract.

Enterobacter spp. that are opportunistic pathogens in humans, fish and other animals [35–38] have been found in the intestines of giant pandas [8, 39]. However, to our knowledge their resistance to antimicrobials has not been investigated. Compared to our E. coli isolates, the rates of resistance to ampicillin and amoxicillin were higher among the Enterobacter spp. isolates. Enterobacter spp. carry resistance genes that promote the MDR phenotype [40–43], it could be due to their ability to acquire numerous genetic mobile elements containing resistance genes [44], making them a potential problem for giant pandas. Unlike the Enterobacter spp. strains from humans and companion animals [45, 46], the giant panda Enterobacter spp. isolates were not resistant to ciprofloxacin, indicating that quinolone antimicrobials may remain effective in treating Enterobacter infections [47].

Over 70% of the K. pneumoniae isolates were resistant to ampicillin and amoxicillin. The prevalence of ESBL-producing K. pneumoniae in many areas of the world has reached 50%, indicating that its antimicrobial resistance is ubiquitous [48]. In Asia, the prevalence of resistance to most of the commonly used antimicrobials is high among K. pneumoniae [49]. In China, the probabilities of MDR K. pneumoniae infections are high, so management of antimicrobial resistance in MDR K. pneumoniae has been a major challenge for clinical veterinarians. K. pneumoniae may play a key role in disseminating ARGs from environmental microbes to clinically important pathogens because of its wider ecological distribution, greater ARG diversity or a higher mobile genetic element burden than other Gram-negative opportunists [50, 51].

Studies on the antimicrobial resistance of Enterococcus spp. derived from giant pandas are few. In our study, the Enterococcus isolates were mainly resistant to tetracycline, erythromycin and ampicillin. Compared with the giant pandas, the rate of tetracycline resistance among wild rabbit-derived Enterococcus spp. was higher [52], possibly due to the contamination of water or vegetation in the woodlands by fecal material from wild birds or even humans [53]. The intrinsic resistance of Enterococcus spp. to semisynthetic penicillin, aminoglycosides, vancomycin, polymyxins and streptogramins has compromised the choice of therapeutic options for the treatment of enterococcal infections [54]. It is suggested that when treating Enterococcus infections, antimicrobials should be selected according to the susceptibility and resistance among the isolates to reduce the generation of antimicrobial-resistant strains and the spread of antimicrobial-resistance genes.

The only difference between the isolates from female and male giant pandas was the lower TET resistance rate in Enterococcus isolates from females. In comparing the giant panda age groups, the differences in the resistance rates among E. coli, K. pneumoniae and Enterobacter spp. isolates suggested that the infections in giant pandas of different age should be treated differently. Diet conversion from infancy to adolescence may induce higher prevalence of gastroenteritis that is treated with antimicrobials causing high antimicrobials-resistance rate [55]. In our study, the resistance prevalence to some antimicrobials were higher among the isolates from the infant giant pandas or the old giant pandas than in the other age groups. At the age of 7–18 months, the diet of the giant pandas changes gradually from breast milk or artificial milk to bamboo, which can lead to intestinal diseases and affect the health of the pandas [56]. The probability of intestinal infection is higher at old age because of weakened immunity, basic diseases and long-time application of wide-spectrum antimicrobials [20]. For the K. pneumoniae and E. coli isolates, the prevalence of resistance to sulfadiazine was highest and lowest, respectively, among isolates from adult pandas. The difference may

![Fig. 5](image-url) The structure of the class 1 integron resistance gene cassette in the genome of K. pneumoniae KBS. Genes encoding antimicrobial resistance are indicated with red and mobile genetic elements with yellow.
be associated with differences in resistance mechanisms, spread of resistance genes or in inherent characteristics of the taxa, yet further research is needed to confirm the cause.

*Enterobacter* isolates are able to produce extended-spectrum β-lactamases of *CTX-M* _TEM_ and _SHV_ types, and β-lactamases are the prominent reason for β-lactam resistance in most *Enterobacter* species [44]. The _bla_ _TEM_, _bla_ _SHV_ and _bla_ _CTX-M_ genes that have been found in *Enterobacter* spp. isolates from other animals, including humans [57, 58], were detected in the isolates from the giant pandas as well. The differences in the prevalence of the _bla_ types implied that the genetic basis for β-lactam resistance among the *E. coli, Enterobacter* spp. and *K. pneumoniae* isolates were different.

The genome of *K. pneumoniae* K85, an isolate resistant to sixteen antimicrobials, contained multiple ARGs. Efflux pump genes were the most numerous ARGs, indicating that the efflux pumps are the main determinants for the resistance. Efflux pumps are commonly found in bacteria and mediate resistance to antimicrobials, disinfectants, detergents and dyes [59]. Overexpression of the efflux pump genes can lead to multi-drug resistance: the efflux pump encoded by _emrE_ can pump tetracycline, erythromycin, crystal violet and the stain ethidium bromide [60], and the pump encoded by _mdfA_ can pump ciprofloxacin, kanamycin, neomycin, and quaternary ammonium disinfectants out of cells [61]. Even though *K. pneumoniae* K85 was resistant to all β-lactams except aztreonam, the genome of *K. pneumoniae* K85 contained the resistance gene _bla_ _CTX-M-3_ that encodes an aztreonam hydrolyzing enzyme [62]. In addition, we detected mobile genetic elements including insertion sequences, integrons and plasmids that can mobilize antimicrobial resistance genes. The insertion sequence _ISEcp1_, adjacent to the _bla_ genes in the K85 genome, is associated with the expression and mobilization of _bla_ _CTX-M_ genes [63, 64]. Thus, the location of insertion sequences and integron-integrase genes next to the resistance genes in the genome of *K. pneumoniae* K85 implied a potential for gene transfer between different plasmids.

**Conclusions**

In summary, the *E. coli, Enterobacter* spp., *K. pneumoniae* and *Enterococcus* spp. isolated from the feces of giant pandas showed resistance to various antimicrobials and carried several ARGs, implying that the gut bacteria may pose serious health risks for captive giant pandas. The resistance genes in the genome of *K. pneumoniae* K85 were associated with insertion sequences and integron-integrase genes, implying a potential for the further spread of the antimicrobial resistance.

**Materials and methods**

**Bacterial isolation and identification**

Fresh feces of 166 giant pandas were sampled in May to June 2018, including eight infant giant pandas (aged <1.5 years), 51 adolescent giant pandas (aged 1.6 to 5 years), 98 adult giant pandas (aged 6 to 20 years) and nine old giant pandas (aged >21 years) (Supplementary Table S2). Twenty-five-gram samples were taken aseptically, placed in sterile conical flasks with 225 mL of buffered peptone water (BPW; HuanKai Microbial Technology Co., Ltd., Guangzhou, China) and incubated for 16–18 h at 200 rpm at room temperature. One loopful of overnight BPW culture was streaked onto MacConkey agar (MAC) and eosin methylene blue agar (EMB), Simmons Citrate Agar (SCA) and Pfizer Selective *Enterococcus* Agar (EA) (HuanKai Microbial Technology Co., Ltd., Guangzhou, China), and incubated at 37°C for 18–24 h. Typical *E. coli* colonies (large, blue-black and green metallic sheen) on EMB, *K. pneumoniae* colonies (the agar turns to blue) on SCA, *Enterococcus* spp. colonies (brown-black colony with brown-black halo) on EA and other colonies on EMB were streaked onto Soybean Casein Digest Agar (TSA, HuanKai Microbial Technology Co., Ltd., Guangzhou, China). Isolates were purified using standard methods and grown in Tryptic Soy Polymyxin Broth Base (TSB; HuanKai Microbial Technology Co., Ltd., Guangzhou, China) at 37°C. After Gram-staining, Matrix-Assisted Laser Desorption/Ionisation Time of Flight Mass Spectrometry (MALDI-TOF-MS/ Autoflex speed TOF/TOF, Bruker, Germany) [65] was used for identification. The 166 *E. coli, 68 Enterobacter* spp., 116 *K. pneumoniae* and 117 *Enterococcus* spp. isolates were stored in TSB containing 25% glycerol at −80°C.

**Antimicrobial susceptibility testing**

Susceptibility to antimicrobials was determined in triplicate using the standard agar dilution method recommended by the Clinical and Laboratory Standards Institute (CLSI, 2020) [66]. The following eighteen antimicrobials were tested: kanamycin (KAN), gentamicin (GEN), erythromycin (ERY), azithromycin (AZM), norfloxacin (NOR), ofloxacin (OFX), ciprofloxacin (CIP), lomefloxacin (LOM), levofloxacin (LEV), sulfadiazine (SD), trimethoprim (TMP), ceftriaxone (CRO), cefixime (CFM), ampicillin (AMP), amoxicillin (AML), aztreonam (ATM), imipenem (IPM) and tetracycline (TET) (Meilun Biotechnology Co., LTD, Dalian, China). The isolates were grown on TSA plates, suspended in stroke-physiological saline solution to a turbidity equivalent to 0.5 McFarland Standard, and inoculated onto Mueller-Hinton agar plates using a multipoint inoculator (MIT-60P; Sakuma Seisakusyo, Tokyo, Japan). The final inoculum
DNA extracts were stored at 20 °C. Antibiotic resistance was estimated with a NanoDROP ONE (Thermo Scientific, USA). The concentration and purity of the extracted DNA was estimated by spectrophotometry at 260/280 nm. DNA was extracted by suspending an overnight culture grown on TSA in 600 μl of reagent-grade water, incubating the suspension at 100 °C for 10 min, centrifuging at 11000 g for 5 min and collecting the supernatant. The concentration and purity of the extracted DNA was estimated with a NanoDROP ONE (Thermo Scientific, USA) and a Qubit3.0 system (Life InVitrogen, USA). DNA extracts were stored at −20 °C. Antibiotic resistance genes were amplified using primers and amplification conditions as described previously [2, 20, 22, 67–73] (Supplementary Table S4). Amplification products were assessed using electrophoresis in 1.0% (w/v) agarose gel. All results were confirmed by at least two independent experiments. Confirming that the amplification products were the target resistance genes was done using Sanger sequencing.

Detection of antimicrobial resistance genes
DNA was extracted by suspending an overnight culture grown on TSA in 600 μl of reagent-grade water, incubating the suspension at 100 °C for 10 min, centrifuging at 11000 g for 5 min and collecting the supernatant. The concentration and purity of the extracted DNA was estimated with a NanoDROP ONE (Thermo Scientific, USA) and a Qubit3.0 system (Life InVitrogen, USA). DNA extracts were stored at −20 °C. Antibiotic resistance genes were amplified using primers and amplification conditions as described previously [2, 20, 22, 67–73] (Supplementary Table S4). Amplification products were assessed using electrophoresis in 1.0% (w/v) agarose gel. All results were confirmed by at least two independent experiments. Confirming that the amplification products were the target resistance genes was done using Sanger sequencing.

Whole-genome sequencing of K. pneumoniae
Genomic DNA of K. pneumoniae K85 was extracted using an UltraClean1 Microbial DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA). The concentration and purity of the extracted DNA was estimated as described above. The genome of K. pneumoniae K85 was sequenced using Illumina NovaSeq PE150 at the Beijing Novogene Bioinformatics Technology Co., Ltd. (Beijing, China). The Raw data was filtered to obtain valid data (Clean Data). The sequences were assembled using SOAPdenovo (version 2.04) [74, 75], SPAdes [75] and ABySS [76], the assemblies were integrated with CISA [77] with default parameters. Then filling the gaps of preliminary assembly results, fragments below 500 bp were filtered out and the final result was counted for gene prediction. Antimicrobial resistance genes were predicted using the Comprehensive Antibiotic Research Database (CARD, https://card.mcmaster.ca) with default BLAST expectation value \( e^{-30} \) and annotated with the highest estimate (default identity ≥40%, coverage ≥40%). The sequences were compared using BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and EasyFig 2.2.7 with default parameters [78]. Plasmid type analysis was done using database Enterobacteriales in Plasmid Finder v.2.0 with 100% identity and 60% coverage (https://cge.cbs.dtu.dk/services/PlasmidFinder/) [79].

Data analysis
Statistical testing of the differences was tested using \( \chi^2 \) test of independence or Fisher’s exact test in IBM SPSS Statistics 26 software with default parameters [20]. A \( P \)-value < 0.05 was considered statistically significant. Other statistical analyses were done using Microsoft Excel (Microsoft, Inc., Washington DC, USA).

Additional file 1: Figure S1. The proportion of antimicrobial resistant isolates from female and male giant pandas. KAN, kanamycin; GEN, gentamicin; AZM, azithromycin; ERY, erythromycin; NOR, norfloxacin; OFX, ofloxacin; CIP, ciprofloxacin; LOM, lomefloxacin; LEV, levofloxacin; SD, sulfadiazine; TMP, trimethoprim; CRO, ceftriaxone; CFX, cefixime; AMP, ampicillin; AML, amoxicillin; ATM, aztreonam; IPM, imipenem; TET, tetracycline; CLSI: The Clinical and Laboratory Standards Institute; CARD: The Comprehensive Antibiotic Research Database.

Supplementary Information
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Additional file 2: Table S1. Antimicrobial resistance profiles of the 144 E. coli, 66 Enterobacter spp., 110 K. pneumoniae and 43 Enterococcus spp. isolates from the feces of giant pandas in China. Table S2 The location, sex and age of the sampled giant pandas. Table S3 MIC breakpoints for Enterobacteriales and Enterococcus spp. according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. Table S4 Primers, expected product sizes and annealing temperatures in the amplification of antimicrobial resistance genes.

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Authors’ contributions
Xin Wang, Yi Zhang and Caiwu Li contributed equally to this work. All authors approved the manuscript’s final version.

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Availability of data and materials
All data generated and analyzed in this study are included in this published article and the supplementary materials. The assembly genome sequencing data for K. pneumoniae strain K88 were deposited at CGNB Sequence Archive (CNSA) of China National GeneBank DataBase (CNGBdb) with accession number: CNP0002458 (https://db.cngb.org/cnsa/project/page/sub026616).

Abbreviations
GPs: Giant pandas; AMR: Antimicrobial resistance; ARGs: Antibiotic resistance genes; MDR: Multidrug-resistant; ESRLs: Extended-spectrum \( \beta \)-lactamases; BPW: Buffered peptone water; MAC: MacConkey agar; EMB: Eosin methylene blue agar; SCA: Simmons Citrate Agar; EA: Pfizer Selective Enterococcus Agar; TSA: Tryptic Soy Agar; MALT-TOF-MS: Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry; KAN: Kanamycin; GEN: Gentamicin; ERY: Erythromycin; AZM: Azithromycin; NOR: Norfloxacin; OFX: Ofloxacin; CIP: Ciprofloxacin; LOM: Lomefloxacin; LEV: Levofloxacin; SD: Sulfadiazine; TMP: Trimethoprim; CRO: Ceftriaxone; CFX: Cefixime; AMP: Ampicillin; AML: Amoxicillin; ATM: Aztreonam; IPM: Imipenem; TET: Tetracycline; CLSI: The Clinical and Laboratory Standards Institute; CARD: The Comprehensive Antibiotic Research Database.
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