Antimicrobial Resistance and Cytotoxicity of Citrobacter spp. in Maanshan Anhui Province, China

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Objectives: Citrobacter spp. especially Citrobacter freundii, is frequently causing nosocomial infections, and increasingly becoming multi-drug resistant (MDR). In this study, we aimed to determine the genetic diversity and relationships of Citrobacter spp. from diarrheal patients and food sources, their antimicrobial resistance profiles and in vitro virulence properties.

Methods: Sixty two Citrobacter isolates, including 13 C. freundii and eight C. braakii isolates, were obtained from human diarrheal patients and food sources. Multilocus Sequence Typing (MLST) of seven housekeeping genes and antimicrobial susceptibility testing using the broth microdilution method according to CLSI recommendations were carried out. Adhesion and cytotoxicity to HEp-2 cells were performed. PCR and sequencing were used to identify \textit{bla}_{CTX-M}, \textit{bla}_{SHV}, \textit{bla}_{TEM} and \textit{qnr} genes.

Results: The 62 isolates were divided into 53 sequence types (STs) with all STs being novel, displaying high genetic diversity. ST39 was a predominant ST shared by 5 C. youngae strains isolated from four foods and a diarrheal patient. All isolates were resistant to cefoxitin, and sensitive to imipenem, meropenem and amikacin. The majority of Citrobacter isolates (61.3\%) were MDR of three or more antibiotics out of the 22 antibiotics tested. Two C. freundii isolates each carried the \textit{bla}_{TEM-1} gene and a variant of \textit{qnrB77}. Three Citrobacter isolates each carried \textit{qnrS1} and \textit{aac(6')-Ib-cr} genes. Seven isolates that showed strong cytotoxicity to HEp-2 cells were MDR.

Conclusions: Citrobacter spp. from human and food sources are diverse with variation in virulence properties and antibiotic resistance profiles. Food may be an important source of Citrobacter species in transmission to humans. C. freundii and C. youngae are potential foodborne pathogens.

Keywords: Citrobacter, Multilocus sequence typing, Multidrug resistance, adhesion, cytotoxicity
INTRODUCTION

*Citrobacter* spp. are commensal inhabitants of the intestinal tract of humans and other animals. They have also been recovered from water, sewage, and soil (Nada et al., 2004; Bae et al., 2010). *Citrobacter* spp. are opportunistic pathogens of humans and have been associated with a range of infections including urinary tract infections (UTIs), gastroenteritis, wound infections, pneumonia, brain abscesses, septicaemia, meningitis, and endocarditis, in particular in neonates and immunocompromised hosts (Doran, 1999). *Citrobacter freundii* is the most common *Citrobacter* species causing infections (Mohanty et al., 2007; Samonis et al., 2009; Bai et al., 2012), *C. youngae* and *C. braakii* are rarely a cause of infections. Some *C. freundii* isolates have acquired virulence traits and caused food poisoning or diarrhea in humans (Bai et al., 2012). The main virulence factors found in diarrhea-associated *C. freundii* are toxins, including Shiga-like toxins, heat stable toxins and a cholera toxin B subunit homolog (Bae et al., 2010). In our previous study, we identified one cytotoxic and aggregative *C. freundii* strain and found strains causing diarrheal infections in humans belonged to four sequence types (STs) (Bai et al., 2012). *C. braakii* has been associated with infections, such as hospital-acquired bacteremia and UTIs, making it an opportunistic pathogen (Arens and Verbist, 1997). It was reported that *C. braakii* caused an acute peritonitis in peritoneal dialysis patients (Bai et al., 2012). Moreover, *C. braakii* has been isolated from raw ground beef samples and pork products (Basra et al., 2015; Kwak et al., 2015).

*Citrobacter* spp. as a bacterial contaminant, has been partly responsible for the cause of food-borne diseases, and often transmitted through food and water (Ifeadike et al., 2012). Accordingly, food-handlers with poor personal hygiene could be potential sources of infections by these microorganisms (Ifeadike et al., 2012; Settanni et al., 2013). *Citrobacter* has been isolated from a range of foods (Tassew et al., 2010; Saba and Gonzalez-Zorn, 2012; Kouamé et al., 2013) and food poisoning and diarrhea caused by foods contaminated by *Citrobacter* had been reported (Warner et al., 1991; Tschape et al., 1995; Doulgeraki et al., 2011; Giammanco et al., 2011).

Extended spectrum β-lactamases (ESBLs) producing *Citrobacter* strains have been reported. The prevalence of ESBLs varied among countries and *Citrobacter* spp. with reports of 4.9–20.6%, 0.2–4.6%, and 0.9% of *C. freundii* isolates from Korea, Japan and USA, respectively; and 3.5 and 60.0% of *C. koseri* isolates from USA and Japan, respectively (Park et al., 2005; Moland et al., 2006; Choi et al., 2007). Among *Citrobacter* spp. various CTX-M types, SHV and TEM have been reported worldwide (Kanamori et al., 2011).

Plasmid-mediated quinolone resistance genes including *qnr* and *aac(6’)-Ibcr* have been reported in *Citrobacter* spp. (Park et al., 2007; Zhang et al., 2012). The *qnr* and *aac(6’)-Ibcr* genes were present in 72.8 and 11.6% of clinical *C. freundii* isolates from China, respectively (Zhang et al., 2012). The prevalence of *qnr* genes was found in 38.4% of *C. freundii* isolates in Korea (Park et al., 2007). Numerous *qnrB* alleles have been detected, which seem to be more common than other *qnr* genes (Jacoby et al., 2014). About 40 *qnrB* variants are located on the chromosome of *Citrobacter* spp. especially *C. freundii* (Liao et al., 2015). Of the clinical *C. freundii* isolates with the *qnr* gene, 63.1% carried *qnrB* (Bae et al., 2010).

In this study, we analyzed the genetic diversity by Multilocus Sequence Typing (MLST) and antimicrobial resistance profiles of *Citrobacter* isolates from diarrheal patients, food and food-handlers in Maanshan Anhui Province, China, investigated the prevalence of *blaCTX-M*, *blaSHV*, *blaTEM* and *qnr* genes and determined the adhesion and cytotoxicity to HEP-2 cells of the isolates.

MATERIALS AND METHODS

Ethics Statement

This study was reviewed and approved by the ethics committee of National Institute for Communicable Disease Control and Prevention, China CDC. Human fecal specimens were acquired with the written informed consent of the diarrheal patients and food-handlers with the approval of the ethics committee of National Institute for Communicable Disease Control and Prevention, according to the medical research regulations of Ministry of Health (permit number 2007-17-3).

**Citrobacter** Isolates

Sixty two *Citrobacter* isolates, including 13 *C. freundii*, eight *C. braakii* and 41 *C. youngae* isolates were obtained from patients and food samples from 2007 to 2011 in Maanshan Anhui Province, China. Among these 62 isolates, 18 *C. youngae* and two *C. freundii* isolates were obtained from diarrheal patients. The diarrheal patients harbored no other known enteric bacterial pathogens. Viral causes were not investigated. 42 isolates, including 23 *C. youngae*, 11 *C. freundii* and eight *C. braakii* were isolated from foods (including chicken, pork, fish and vegetables) and food-handlers (Table 1). The identity of each isolate was determined using API 20E test strips (bioMérieux, La Balme les Grottes, France) at the time of isolation, and they were stored as glycerol stocks at –80°C. Bacteria were grown in Luria-Bertani (LB) broth or on LB and Mueller–Hinton agar plates (pH 7.4) at 37°C.

**Multilocus Sequence Typing and Phylogenetic Analysis**

The *Citrobacter* MLST scheme (http://pubmlst.org/cfrendii/) was used. The seven housekeeping genes for MLST were *aspC*, *clpX*, *fadD*, *mdh*, *arcA*, *dnaG* and *lysP*, and the MLST primers were as previously described (Bai et al., 2012) and synthesized by Shanghai Sangon Biological Engineering Technology and Services (Shanghai, China). PCR products were verified on 1% agarose gels and purified. DNA sequence was determined using Sanger sequencing in both directions (Shanghai Sangon Biological Engineering Technology and Services, China). Sequences were analyzed using SeqMan 7.0 software.

**Antimicrobial Susceptibility Testing**

Antimicrobial susceptibility testing was carried out using the broth microdilution method according to CLSI...
### TABLE 1 | Citrobacter isolates used in this study and their characteristics.

| Isolates | Species     | STs | Year | Source        | Adhesion | LDH          |
|----------|-------------|-----|------|---------------|----------|--------------|
| AH2007001 | C. youngae  | 25  | 2007 | Diarrheal patient | **       | 22.4 ± 1.1   |
| AH2007002 | C. youngae  | 25  | 2007 | Diarrheal patient | **       | 9.8 ± 0.7    |
| AH2007003 | C. youngae  | 26  | 2007 | Diarrheal patient | **       | 5.9 ± 0.1    |
| AH2007004 | C. youngae  | 27  | 2007 | Diarrheal patient | **       | 37.1 ± 2.6   |
| AH2007006 | C. youngae  | 28  | 2007 | Diarrheal patient | ***      | 24.1 ± 0.5   |
| AH2007007 | C. youngae  | 28  | 2007 | Diarrheal patient | **       | 8.1 ± 0.5    |
| AH2007008 | C. youngae  | 29  | 2007 | Diarrheal patient | **       | 22.3 ± 1.8   |
| AH2007009 | C. youngae  | 30  | 2007 | Diarrheal patient | **       | 3.7 ± 1.2    |
| AH2007010 | C. youngae  | 31  | 2007 | Diarrheal patient | **       | 0.1 ± 1.3    |
| AH2007013 | C. youngae  | 32  | 2007 | Diarrheal patient | **       | 3.2 ± 0.8    |
| AH2007014 | C. freundii | 33  | 2007 | Diarrheal patient | **       | 5.3 ± 0.8    |
| AH2007015 | C. youngae  | 34  | 2007 | Diarrheal patient | **       | 18.7 ± 6.4   |
| AH2007016 | C. youngae  | 35  | 2007 | Diarrheal patient | *        | 1.3 ± 0.5    |
| AH2007018 | C. youngae  | 37  | 2007 | Diarrheal patient | *        | 16.7 ± 4.1   |
| AH2007021 | C. youngae  | 38  | 2007 | Diarrheal patient | *        | 4.2 ± 4.2    |
| AH2007022 | C. youngae  | 39  | 2007 | Diarrheal patient | **       | 11.5 ± 1.3   |
| AH2007023 | C. youngae  | 40  | 2007 | Food-handler    | **       | 21.4 ± 5.8   |
| AH2007024 | C. youngae  | 39  | 2007 | Food-handler    | **       | 6.6 ± 0.4    |
| AH2007025 | C. youngae  | 39  | 2007 | Food-handler    | **       | 11.9 ± 0.2   |
| AH2007026 | C. youngae  | 41  | 2007 | Food-handler    | **       | 0.1 ± 0.5    |
| AH2008001 | C. youngae  | 39  | 2008 | Beef           |          | 36.5 ± 2.4   |
| AH2008002 | C. youngae  | 39  | 2008 | Egg            | *        | 3.3 ± 0.4    |
| AH2008004 | C. freundii | 42  | 2008 | Carp meat      | **       | 19.3 ± 1.3   |
| AH2008005 | C. freundii | 43  | 2008 | Duck leg       | —        | 14.9 ± 7.8   |
| AH2008006 | C. freundii | 44  | 2008 | Carp meat      | **       | 0.1 ± 0.4    |
| AH2008007 | C. freundii | 45  | 2008 | Flower silver carp | *     | 0.7 ± 0.4    |
| AH2008008 | C. freundii | 46  | 2008 | Duck leg       | **       | 11.0 ± 2.0   |
| AH2008009 | C. freundii | 47  | 2008 | Pigeon meat    | ***      | 20.2 ± 5.4   |
| AH2008010 | C. youngae  | 48  | 2008 | Carp meat      | **       | 29.2 ± 2.3   |
| AH2008011 | C. youngae  | 49  | 2008 | Chicken breast | *        | 5.7 ± 0.2    |
| AH2008012 | C. youngae  | 50  | 2008 | Anchovy        | **       | 9.2 ± 0.2    |
| AH2008014 | C. braakii  | 51  | 2008 | Duck neck      | —        | 4.4 ± 1.8    |
| AH2008015 | C. braakii  | 52  | 2008 | Food-handler   | ±        | 11.0 ± 4.6   |
| AH2008016 | C. youngae  | 53  | 2008 | Food-handler   | ±        | 4.4 ± 0.7    |
| AH2009001 | C. freundii | 54  | 2009 | Pork liver     | ±        | 2.2 ± 1.2    |
| AH2009002 | C. braakii  | 55  | 2009 | Carp meat      | *        | 1.7 ± 0.1    |
| AH2009003 | C. youngae  | 56  | 2009 | Carp meat      | **       | 14 ± 0.6     |
| AH2009004 | C. youngae  | 57  | 2009 | Pork           | **       | 5.2 ± 0.2    |
| AH2009006 | C. braakii  | 58  | 2009 | Meat           | **       | 0.5 ± 0.1    |
| AH2009007 | C. youngae  | 59  | 2009 | Meat           | **       | 19.8 ± 3.9   |
| AH2009008 | C. youngae  | 60  | 2009 | Catfish        | *        | 0.1 ± 0.4    |
| AH2009009 | C. youngae  | 59  | 2009 | Chicken thigh  | *        | 13.5 ± 2.3   |
| AH2009010 | C. youngae  | 71  | 2009 | Pork           | ***      | 60.4 ± 2.7   |
| AH2009011 | C. youngae  | 72  | 2009 | Meat           | **       | 29.4 ± 3.8   |
| AH2009012 | C. youngae  | 59  | 2009 | Pomfret        | ±        | 4.8 ± 0.8    |
| AH2009013 | C. youngae  | 73  | 2009 | Diarrheal patient | ±     | 13.2 ± 0.7   |
| AH2009014 | C. youngae  | 74  | 2009 | Diarrheal patient | ±     | 1.4 ± 0.7    |
| AH2009015 | C. youngae  | 75  | 2009 | Diarrheal patient | *     | 15.2 ± 2.8   |
| AH2009016 | C. youngae  | 76  | 2009 | Fish heads     | —        | 0.1 ± 0.1    |
| AH2009017 | C. youngae  | 77  | 2009 | Yellow-fin tuna | *       | 2.2 ± 1.1    |
recommendations. Minimum inhibitory concentration (MIC) results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. The antibiotics were serially diluted 2-fold in 50 μL of cation-adjusted Mueller-Hinton broth. The bacterial suspension was prepared from actively growing bacteria in 5 mL of cation-adjusted Mueller-Hinton broth, and diluted to a bacterial cell density of 10^6 colony forming units (CFU)/mL. Five microliter of bacterial suspension was then added to wells containing 100 μL of serially diluted antimicrobial agents to yield a final inoculum of approximately 5 × 10^4 CFU/mL. The MICs were read after overnight incubation (18–24 h) at 35°C. Quality control for MICs was performed using the reference E. coli ATCC 25922.

**PCR Amplification and Sequencing**

All the isolates were screened for the following genes, *qnrA*, *qnrB*, *qnrS*, *qnrC*, *qnrD*, 6'-Ib-cr, *gepA*, *blaCTX-M*, *blaSHV*, and *blaTEM* by PCR using primers listed in Table S1. Primers of *qnrA*, *qnrB*, *qnrS*, *qnrC*, *qnrD*, 6'-Ib-cr, and *gepA* were from Shao et al. (Shao et al., 2011), primers for screening *blaCTX-M*, *blaSHV* and *blaTEM* genes were from Zhang et al. (2011). All primers were synthesized by Shanghai Sangon Biological Engineering Technology and Services (Shanghai, China). Positive PCR products were confirmed by sequencing.

**In vitro Adhesion and Cytotoxicity Assays**

*In vitro* adhesion to host cells was performed using the human epidermoid carcinoma cell line HEp-2 (CCC0068; Beijing Union Medical College cell resource center), as previously described (Bai et al., 2012). An adhesion index (<1; >1 and <50; >50) describing the mean number of bacteria per HEp-2 after examination of 10 visual fields was determined (Bai et al., 2012). Infections were repeated three times in duplicate.

The lactate dehydrogenase (LDH) released by the HEp-2 cells was determined using the Cytotox96 kit (Promega) according to the manufacturer’s instructions. The relative amount of cytotoxicity was expressed as follows: (experimental release–spontaneous release)/(maximum release–spontaneous release)X100, where the spontaneous release was the amount of LDH activity in the supernatant of uninfected cells and the maximum release was that when cells were lysed with the lysis buffer provided by the manufacturer. All experiments were performed two times in duplicate (Bai et al., 2012).

**RESULTS**

**Multilocus Sequence Typing of *Citrobacter* Isolates**

The 62 *Citrobacter* isolates including 13 *C. freundii*, 41 *C. youngae* and eight *C. Braakii* isolates were divided into 53 STs by MLST (Table 1). The 41 *C. youngae* isolates were divided into 32 STs, 13 *C. freundii* isolates into 12 STs and eight *C. Braakii* isolates into 8 STs. Four STs (ST25, ST28, ST39, and ST59), all belonging to *C. youngae*, contained multiple isolates from two to five isolates. ST25 and ST28 each contained two isolates from diarrheal patients. ST39 contained five isolates with one from a diarrheal patient and four from foods. All three ST59 isolates were from foods.

A phylogenetic tree for the 62 isolates and representative isolates for ST1 to ST6 reported previously (Bai et al., 2012) was constructed using the neighbor-joining algorithm based on the concatenated sequences of the seven housekeeping genes (Figure 1). *Salmonella* LT2 was used as an outgroup. The tree could be divided into four clusters with robust bootstrap support of the major divisions. Cluster 1 is comprised of all *C. freundii* isolates; cluster 2 is comprised of all *C. braakii* isolates; and Cluster 3 and cluster 4 are comprised of all *C. youngae* isolates. It is interesting to note that clusters 3 and 4 are not grouped together. Rather, cluster 3 is grouped with clusters 1 and 2 with 90% bootstrap support, suggesting that cluster 3 should be a separate species from cluster 4. However, more isolates are needed to get a better understanding of the diversity of these 3 species and their relationships.

**Antimicrobial Susceptibility**

The 62 *Citrobacter* isolates were tested for susceptibility to 22 antibiotics using the broth microdilution method according to CLSI recommendations (Table 2). All were resistant to cefoxitin (CFX), and sensitive to imipenem (IMI), meropenem (MEM) and amikacin (AMI). Non-susceptibility to β-lactams ranged

| TABLE 1 | Continued |
|-----------|-----------|-----------|-----------|-----------|
| Isolates  | Species   | STs       | Year      | Source    | Adhesion | LDH        |
| AH2009018 | *C. braakii* | 78         | 2009      | Pork      | —        | 4 ± 0.5    |
| AH2010001 | *C. braakii* | 79         | 2010      | Carp meat | —        | 0.1 ± 0.5  |
| AH2010002 | *C. youngae* | 80         | 2010      | Pork      | **       | 0.1 ± 1.3  |
| AH2011001 | *C. braakii* | 81         | 2011      | Carp meat | —        | 0.7 ± 0.1  |
| AH2011002 | *C. braakii* | 82         | 2011      | Carp meat | —        | 0.1 ± 0.3  |
| AH2011005 | *C. youngae* | 83         | 2011      | Water     | *        | 6.4 ± 1.9  |
| AH2011006 | *C. freundii* | 84        | 2011      | Flat fish | **       | 3.5 ± 0.4  |
| AH2011007 | *C. freundii* | 85        | 2011      | Catfish   | ±        | 0.2 ± 0.2  |
| AH2011008 | *C. freundii* | 86        | 2011      | Tofu      | *        | 0.1 ± 0.1  |
| AH2011009 | *C. freundii* | 87        | 2011      | Spiced duck | **     | 4.2 ± 1.3  |
| AH2011010 | *C. youngae* | 87        | 2011      | Snake melon salad | * | 15.7 ± 0.1 |

***, **, * correspond to adhesion index of >50, >1, and <50 and <1 respectively. ± means ambivalent or no adhesion, — means no adhesion.
from 0% to 100%; non-susceptibility to the three quinolones tested ranged from 12.9% to 27.4%; and non-susceptibility to other antibiotics included aminoglycosides (0–12.9%), phenicols (12.9%), sulfonamides (12.9–25.8%), tetracyclines (25.8%), and macrolide (3.2%) (Table 2).

Among the 62 Citrobacter isolates tested for MIC to 22 antibiotics, six C. youngae, seven C. freundii, and four C. braakii isolates were highly resistant to NAL, with MICs > 128 µg/mL and were multidrug resistant, with resistance to ≥3 antibiotics. Among 17 NAL resistant isolates, 14 isolates were from food and three were from diarrheal patients. These isolates belonged to different phylogenetic clusters, seven in cluster 1, four in cluster 2, one in cluster 3 and five in cluster 4. Three C. youngae isolates (one in cluster 3 and two in cluster 4) had a CTX MIC of

![FIGURE 1 | Phylogenetic relationships as determined by MLST data. The presence of ESBLs and qnr genes among the Citrobacter isolates is shown on the right. The tree was constructed using neighbor joining algorithm. For each ST, F, D, H, and O indicate isolates from foods, diarrheal patients, food-handlers and animals respectively. Cluster divisions are marked. Numbers on near the nodes are bootstrap values from 1,000 replicates.](image-url)
Therefore, we suggest that 

We tested the 62 isolates for 

Two 

Genes by PCR 

qnr 

Two 

Genes by PCR and sequencing, both 

qnrB5 

and 

qnrB77 

Table 3 

C. youngae 

isolate (AH2010001). One 

C. youngae 

and two 

C. freundii 

isolates (AH2008004 and AH2008007) were found to harbor an 

aac(6′)-Ib-cr 

gene. These two 

C. freundii 

isolates belonged to two different STs (Figure 1 and Table 1). 

Two 

C. freundii 

isolates (AH2008006 and AH2008007) were found to harbor a 

qnrB gene. This 

qnrB allele has two in-phase ATG start codons. Wang et al. reported that two in-phase ATG start codons are present in many 

qnrB alleles ([qnrB1, qnrB3, and 

qnrB5]). However, in 

qnrB2 and 

qnrB4, the first ATG is out of phase with the remainder of the reading frame, the translation may be initiated at the second ATG codon (Wang et al., 2009). If sequence analysis from the ATG at position 37 (the second ATG codon), our 

qnrB allele has an identical 

qnrB sequence as 

qnrB77. But 

qnrB77 (GenBank accession no. KM985470.1) did not contain this 36 bp region. The 36 bp in our 

qnrB allele contained a LexA binding site (Wang et al., 2009). Therefore, we suggest that our 

qnrB allele is a variant of 

qnrB77. 

These two 

qnrB positive isolates AH2008006 and AH2008007 belonged to two different STs, ST44 and ST45, respectively, suggesting that these isolates were epidemiologically unrelated (Figure 1). 



HEp-2 Cell Adherence of Citrobacter Isolates

Adhesion is an essential virulence property of bacterial pathogens. In vitro assays have been widely used to assess this property (Mange et al., 2006). We tested the 62 isolates for adhesion to HEp-2 cells and categorized the extent of adhesion using the adhesive index (Mange et al., 2006) (Table 1). Four isolates (including three 

C. youngae 

and one 

C. freundii 

) showed the strongest adhesion, with adhesion indexes >50. Twenty-five isolates showed intermediate adhesion, with an adhesion index between 1 and 50. Nineteen isolates showed little adhesion, with an adhesion index of <1. The remaining isolates showed ambivalent adhesion or no adhesion. 

The adhesion rate was lower for 

C. braakii 

(25%) than 

C. youngae 

(88%) and 

C. freundii 

(77%). No difference was evident (P > 0.05) when adhesion behavior was compared in view of the source (human and food) of the 

Citrobacter isolates.

HEp-2 Cell Cytotoxicity of Citrobacter Isolates

The 62 

Citrobacter isolates were tested for Cytotoxicity to cultured HEp-2 cells by measuring the amount of lactate dehydrogenase (LDH) released by HEp-2 cells. We tested all isolates at 8 h. The released LDH levels ranged from 0.1–60.0% (Table 3). 

C. freundii strain CF74 was used as a positive control of cytotoxicity and 

C. freundii strain CF72 was used as a negative control (Bai et al., 2012). The levels of LDH released by CF74 and CF72 were 25.7 and 12.8% respectively. Seven isolates (including five 

C. youngae 

and two 

C. freundii isolates) released LDH more than 24%, showing strong cytotoxicity (Table 1). Among these seven isolates, three isolates showed strongest adhesion;
| Isolates     | Source | Antibiotics |
|--------------|--------|-------------|
|              |        | AMP  | AZM | FEP | CAZ | CLP | LEV | SXT | CTX | TIO | NAL | CHL | STR | SUL | TET | AMZ | KAN | DOX |
| AH2007001    | D      | 16   | 32  |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2007002    | D      | 32   | >8/152 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2007003    | D      | 32   | >32  |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2007004    | D      | 32   | 4    | 8   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2007006    | D      | 32   | >32  | >16 | >32 | >128 | >32 | >64 | >64 | 8  |     |     |     |     |     |     |     |     |
| AH2007007    | D      | 32   | >8/152 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2007008    | D      | 32   | 16   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2007009    | D      | 32   | >32  | >32 | >16 | >32 | >32 | >16 |     |     |     |     |     |     |     |     |     |     |
| AH2007010    | D      | 32   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2007013    | D      | 32   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2007014    | D      | 32   | 8    | >128 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2007015    | D      | 32   | >8/152 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2007016    | D      | 32   | >8/152 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2007018    | D      | 32   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2007019    | D      | 32   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2007021    | D      | 64   | 16   | >128 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2007022    | D      | 64   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2007023    | F      | 32   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2007024    | F      | 32   | 16   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2007025    | F      | 64   | 32   | >32 | >16 | >32 | >32 | 32  | >16 |     |     |     |     |     |     |     |     |     |
| AH2007026    | H      | >128 | 16   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2008001    | F      | 32   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2008002    | F      | 32   | 16   | 4   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2008004    | F      | >128 | >32  | >16 | >8/152 | 4  | >128 | >32 | >32 | >512 | >32 | 64  | >64 | >16 |     |     |     |     |
| AH2008005    | F      | 32   | 16   | >128 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2008006    | F      | 32   | 16   | >128 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2008007    | F      | 32   | 16   | >128 | >32 |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2008008    | F      |     |     | >8/152 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2008009    | F      |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2008010    | F      | >128 | 8    | >16 | >8/152 | >128 | >32 | >32 | >512 | >32 | 64  | >64 | >16 |     |     |     |     |
| AH2008011    | F      | 32   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2008012    | F      |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2008014    | F      | 32   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2008015    | H      | 32   |     | >8/152 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2008016    | H      | 32   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2009001    | F      | 32   | 8    | >16 | >8/152 | 8  | >128 | >32 | >32 | >512 | >32 | 16  |     |     |     |     |     |
| AH2009002    | F      | 32   | 16   | >128 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2009003    | F      | >128 | 32  |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2009004    | F      | 32   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2009006    | F      |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2009007    | F      | 32   | 16   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2009008    | F      |     |     | >8/152 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2009009    | F      |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2009010    | F      | 8    | 16   | >8/152 | >128 | >32 | >32 | >32 | >32 | >32 | >64 | >16 |     |     |     |     |     |
| AH2009011    | F      | 32   | 4    | 16   | >8/152 | >128 | >32 | >32 | >512 | >32 | >64 | >16 |     |     |     |     |     |
| AH2009012    | F      |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2009013    | D      | 64   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2009014    | D      | 32   |     | >8/152 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2009015    | D      | 32   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |

(Continued)
TABLE 3 | Continued

| Isolates     | Source | Antibiotics |
|--------------|--------|-------------|
|              | AMP    | AZM | FEP | CAZ | CLP | LEV | SXT | CTX | TIO | NAL | CHL | STR | SUL | TET | AMZ | KAN | DOX |
| AH2009016    | F      | 32  |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2009017    | F      |     | 4   | 16  | >8/152 | >128 | >32 | >32 | >512 | >32 | >64 | 16  |     |     |     |     |     |     |
| AH2009018    | F      | 32  | 16  |     |     |     | >128 |     |     | >32 |     | 16  |     |     |     |     |     |     |
| AH2010001    | F      | 32  | 8   | 16  | >8/152 | >128 | >32 | >32 | >512 | >32 |     | 16  |     |     |     |     |     |     |
| AH2010002    | F      | 32  | 16  |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2011001    | F      | 32  | 16  |     |     |     | >128 |     |     |     |     |     |     |     |     |     |     |     |
| AH2011002    | F      | 32  | 16  |     |     |     | >128 |     |     |     |     |     |     |     |     |     |     |     |
| AH2011005    | F      | 32  |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2011006    | F      | 32  |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2011007    | F      | 32  | 16  |     |     | >128 | >32 | >32 | >512 | >32 |     |     |     |     |     |     |     |     |
| AH2011008    | F      | 32  | 16  |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2011009    | F      |     |     |     | >8/152 |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AH2011010    | F      | 32  |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |

D, diarrheal patients; H, food-handlers; F, foods; MIC, minimum inhibitory concentration; AMP, ampicillin; CTX, cefotaxime; CAZ, ceftazidime; FEP, cephalothin; TIO, cefotaxim sodium; AZM, aztreonam; NAL, nalidixic acid; CLP, ciprofloxacin; LEV, levofloxacin; CHL, chloramphenicol; STR, streptomycin; SUL, sulfadiazine; TET, tetracycline; SXT, trimethoprim/Trimethoprim; AMZ, azithromycin; KAN, kanamycin; DOX, doxycycline.

Four isolates showed intermediate adhesion (Figure 2). Another seven isolates (including six C. youngae and one C. freundii isolates) released LDH from 18.7 to 22.4% and are considered intermediate cytotoxic. The remaining 48 isolates showed LDH release <16.7% and are likely to be non-cytotoxic.

Seven strongly cytotoxic isolates were multidrug resistant, with resistance to ≥3 antibiotics (Tables 1, 3). Four isolates (AH2008004, AH20080010, AH2009010, and AH2009011) showed multi-drug resistant (MDR) to nine antibiotics (CFX, NAL, CLP, LEV, CHL, STR, TET, SXT, and DOX). Moreover, AH2007006 harbored an aac(6′)-Ib-cr gene, AH2008004 harbored a blatem-1 gene, and an aac(6′)-Ib-cr gene, and AH2008010 harbored a qnsI1 gene.

Four intermediate cytotoxic isolates (including AH2007001, AH2007008, AH2008002, and AH2009007) were resistant to AMP, CAZ and CFX (Tables 1, 3).

DISCUSSION

Citrobacter spp., especially C. freundii, is recognized as an emerging opportunistic pathogen and is known to cause a variety of infections (UTIs, wound infections, gastrointestinal infections, septicaemia, meningitis), especially in immunocompromised patients and in hospital settings (Joaquim et al., 1991; Brenner et al., 1993; Gupta et al., 2003; Samonis et al., 2009; Ranjan and Ranjan, 2013; Leski et al., 2016a). This emergence has coincided with the finding that C. freundii is often resistant to multiple classes of antibiotics, suggesting that both clinical and environmental strains may be a reservoir of antimicrobial resistance determinants (Pepperell et al., 2002; Nada et al., 2004; Yin et al., 2013; Feng et al., 2015; Leski et al., 2016a; Sheppard et al., 2016). A recent survey of outpatients in Bo, Sierra Leone, revealed that a surprisingly high number of C. freundii isolates from UTIs were highly MDR (Leski et al., 2016b). In this study, we surveyed Citrobacter species from diarrheal patients and foods to provide a better understanding of their genetic diversity, antibiotic resistance profile, virulence properties and their potential as foodborne pathogens.

The worldwide prevalence of ESBLs in Citrobacter spp. was reported to be 0.5–36% (Ali et al., 2004; Fernandes et al., 2014; Praharaj et al., 2016). In India, 80.9% of Citrobacter isolates from hospitalized patients were ESBL producers (Praharaj et al., 2016). In this study, we did not test for ESBL phenotype but screened by PCR for Blactx-M, blatem and blashv genes. We found that a very low percentage of our isolates were blatem-1 positive (3.2%) and none carried Blactx-M and blashv. In contrast to a study in India, Shahid (Shahid, 2010) found that Blactx-M, blatem and blashv were found in 67.5%, 40%, and 25% of Citrobacter isolates from human clinical infections, respectively. However, most of our isolates were from food sources.

The prevalence of qnr and aac(6′)-Ib-cr genes varied. A Korean study showed that 38.4% of C. freundii isolates harbored qnr determinants (Park et al., 2007). A study from China showed prevalence of qnr and aac(6′)-Ib-cr genes at 63.3% and 26.7% in C. freundii isolates, respectively (Yang et al., 2008), while another Chinese study showed the prevalence of qnr and aac(6′)-Ib-cr in C. freundii at 72.8% and 68.9%, respectively (Zhang et al., 2012). The latter study also reported the prevalence of qnr and aac(6′)-Ib-cr in C. braakii at 42.9% and 42.9%, respectively (Zhang et al., 2012). We found much lower prevalence of qnr and aac(6′)-Ib-cr genes at 23.1% and 15.4% in C. freundii isolates; 2.4% and 2.4% in C. youngae isolates, and 12.5% and 0% in C. braakii isolates, respectively.

QnrB is the most common of the five qnr families and has the greatest number of allelic variants (Jacoby et al., 2011). We found a variant of qnrB77 in two C. freundii isolates. The variant contained a 36 bp sequence upstream of the qnrB77 start codon with an in-phase ATG codon at the beginning and a LexA binding
site within the sequence, similar to several other qnrB alleles. The study by Wang et al. showed that the LexA binding site renders the qnrB under SOS control leading to its higher expression in response to ciprofloxacin or mitomycin C treatment (Wang et al., 2009). However, it should be noted that the qnrB77 first reported has no upstream sequence available in the GenBank entry and therefore it cannot be ascertained whether the sequence was absent or not reported.

QnrB-carrying C. freundii isolates do not always show high level of quinolone resistance (Zhang et al., 2012). However, our two qnrB-carrying C. freundii had a high MIC for NAL (>128 µg/mL). C. freundii carrying qnrS and aac(6’)-Ib-cr have been shown to have a higher MIC for quinolones (Zhang et al., 2012). Our results are consistent with this observation. One aac(6’)-Ib-cr-carrying C. freundii and three qnrS1-carrying Citrobacter isolates had high MIC of three quinolones (NAL, >128 µg/mL; CLP, >32 µg/mL; LEV, >16 µg/mL).

High prevalence of multidrug resistant Citrobacter has been reported (Moges et al., 2014; Leski et al., 2016b). Moges et al. found that 13 MDR Citrobacter spp. were isolated from waste water in hospital and non-hospital environments (Moges et al., 2014). Twenty-two MDR C. freundii isolates from outpatient urine samples were resistant to >7 antibiotics out of the 11 tested, and 81.8% of the C. freundii isolates produced ESBLs (Leski et al., 2016b). In this study, 61.3% Citrobacter isolates were resistant to ≥3 antibiotics out of the 22 tested, and seven MDR isolates were strongly cytotoxic and four were intermediate cytotoxic. Moreover, two of the seven strongly cytotoxic and MDR isolates (from C. youngae) were obtained from diarrheal patients. The cytotoxic property of these isolates implies that they may cause more severe disease while the MDR properties limit clinical therapeutic options.

Citrobacter youngae is rarely a cause of infections. It has been reported to cause peritonitis (Chen et al., 2013). However, C. youngae has not been recognized as a diarrheal pathogen. We found that 50% of the isolates showed moderate to strong adhesion and 15% of the isolates also showed strong cytotoxicity. Nearly half of the C. youngae isolates were from diarrheal patients. However, not all human isolates were adhesive or cytotoxic. Three of the six adhesive and cytotoxic isolates were obtained from diarrheal patients, suggesting that such strains are likely to cause diarrheal disease. STs from both human and food
isolates were diverse with most STs being only isolated once. However, three STs were isolated more than once. Interestingly one ST was isolated from food as well as from a diarrheal patient. These findings suggest that C. youngae is a potential foodborne diarrheal pathogen.

*Citrobacter freundii* is the most common cause of *Citrobacter* infections (Mohanty et al., 2007; Samonis et al., 2009) and has been implicated in gastroenteritis associated outbreaks (Guerrant et al., 1976; Warner et al., 1991; Tschepe et al., 1995; Doulgeraki et al., 2011; Giammanno et al., 2011) and foodborne outbreaks (Ifeadike et al., 2012; Settanni et al., 2013). We only obtained two isolates from diarrheal patients. Neither isolate was adhesive and one of them was intermittently cytotoxic, questioning its role in diarrhea in these cases. However, five isolates from foods were adhesive or strongly cytotoxic, suggesting that food isolates serve as a potential foodborne pathogen. The STs from this study were compared with six STs (ST1-ST6) from our previous study and 28 STs from the *Citrobacter* MLST database of global isolates, all STs found in this study were novel STs, showing high diversity of *C. freundii* from different regions and countries.

*Citrobacter braakii* is commonly found in water, soil, food, and the intestinal tracts of animals and humans (Basra et al., 2015). *C. braakii* is an opportunistic pathogen and has been isolated from hospital infections and UTIs (Arens and Verbist, 1997). *C. braakii* can cause acute peritonitis in peritoneal dialysis patients (Chao et al., 2013). All eight *C. Braakii* isolates from this study were isolated from foods. It requires further study to determine whether *C. braakii* contributes to diarrheal disease.

**CONCLUSION**

We analyzed 13 *C. freundii*, 41 *C. youngae*, and eight *C. braakii* isolates from Maanshan Anhui Province, China, isolated from human diarrheal patients and foods for their genetic diversity, antibiotic sensitivity and *in vitro* virulence phenotype. The 62 isolates were divided into 53 STs with all STs being novel, displaying high genetic diversity. Half of the isolates were MDR of three or more antibiotics. The *blaTEM−1* gene was detected in two *C. freundii* isolates, while *qnrS1* and *aac(6′)-Ib-cr* genes were detected in three *Citrobacter* isolates, respectively. We found seven isolates that showed strong cytotoxicity to HEp-2 cells, all of which were MDR. We also found a variant of *qnrB77* that contained a LexA site in two *C. freundii* isolates. Our data suggest that food is an important source of *Citrobacter* species in transmission to humans and *C. freundii* and *C. youngae* are potential foodborne pathogens. Further studies are required to determine their public health significance.

**AUTHOR CONTRIBUTIONS**

LyL and JX designed the project; YlW carried out the sampling work; YZ carried out the experiments; YtW, LqL, and RL analyzed data; LyL and RL drafted the manuscript. All authors have read and approved the final version of the manuscript.

**ACKNOWLEDGMENTS**

This work was supported by grants from National Natural Science Foundation of China (No. 81301401).

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01357/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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