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Prevalence, genetic diversity, and antibiotic resistance of enterotoxigenic *Escherichia coli* in retail ready-to-eat foods in China

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ABSTRACT

Enterotoxigenic *Escherichia coli* (ETEC) is an important food-borne pathogen that can cause diarrhoea. The prevalence and characteristics of ETEC in retail ready-to-eat (RTE) foods have not been thoroughly investigated in China. The objective of this study was to investigate the prevalence of ETEC in Chinese retail RTE foods and characterize the ETEC isolates by serotyping, virulence factors testing, antibiotic resistance screening, pulsed-field gel electrophoresis (PFGE), and multilocus sequence typing (MLST). From May 2013 to April 2014, a total of 559 RTE foods were collected from retail markets in 24 cities of China. *E. coli* and ETEC were detected in 219 (39.2%) and 36 (6.4%) of 559 samples, respectively. Cold noodles and cooked meats were the most frequently contaminated with ETEC (12.3% and 9.9%). Among 36 ETEC isolates, 16 were positive for the *st* gene, 8 for the *lt* gene, and 12 for the *lt* + *st* genes. CS6 and CS21 were the most prevalent colonization factors in these isolates. The 6 non-classical virulence genes *ClyA*, *East1*, *EatA*, *Tia*, *TibA*, and *LeoA* were detected in 97.2%, 27.8%, 27.8%, 13.9%, 27.8%, and 5.6% of 36 isolates, respectively. O-antigen serotyping showed that 25 isolates were typeable and 11 were non-typeable. The most common serotypes were O7 and O8. PFGE and MLST molecular typing indicated a high degree of genetic diversity in ETEC isolates. A total of 28 PFGE patterns and 27 sequence types (STs) were identified in 36 isolates. ST10 was the most prevalent ST. The isolates showed high prevalence of resistance to tetracycline (66.7%), ampicillin (61.1%), and trimethoprim-sulfamethoxazole (58.3%). Genotypic results indicated that the isolates carrying similar virulence factors were often genetically related. Our findings indicated that ETEC contamination of Chinese RTE foods poses a potential threat to the consumer, and highlighted the necessary of implementing good hygienic practices.

1. Introduction

Ready-to-eat (RTE) foods such as cooked meat, roasted poultry, and cold vegetable dishes in sauce, are very popular in China because of their good taste and convenience. In recent years, the consumption of these foods has increased dramatically. Thus, the hygiene and safety of these foods has become a major concern for public health. Since RTE foods are edible without additional treatment, the risks of food-borne outbreaks are high if they are improperly handled. In particular, RTE foods have been recognized as potential vehicles of microbial food-borne bacteria (e.g. *Salmonella*, *Listeria monocytogenes*, and *Staphylococcus aureus*) (Chen, Wu, Zhang, Yan, & Wang, 2014; Huong et al., 2010; Seow, Ágoston, Phua, & Yuk, 2012). Therefore, monitoring the microbiological profile of RTE foods is important to ensure the safety of this type of high-risk food.

Escherichia coli is an important indicator of faecal contamination in food and is commonly used to assess the hygienic quality of food products. Enterotoxigenic *E. coli* (ETEC) is a major cause of traveller's diarrhoea (TD) and young children's diarrhoea in developing countries (Pattabiraman, Parsons, & Bopp, 2016; Rivera et al., 2013; Wang et al., 2010). It is estimated that this organism causes hundreds of millions of cases of diarrheal disease each year, particularly in developing countries (Walker, 2015). The consumption of contaminated food remains a major source of ETEC infection, and food-borne outbreaks caused by ETEC have been reported in many countries (Chen et al., 2011; Cho et al., 2013; Gould et al., 2013; MacDonald et al., 2015).

The pathogenicity of ETEC is associated with several virulence factors, the major ones being enterotoxins and colonization factors

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(CFs). ETEC produces two kinds of enterotoxins, heat-labile toxin and heat-stable toxin. Both toxins generate a net secretion of ions and water, resulting in watery diarrhoea by the activation of the cystic fibrosis transmembrane regulator chloride channel due to increased levels of cyclic AMP or cyclic GMP (Fleckenstein et al., 2010; Kaper, Nataro, & Mobley, 2004), and the detection of these enterotoxin genes is usually used to identify this pathogen (Kagambèga et al., 2012; Mohammed, 2012). Additionally, many ETEC strains are able to produce CFs, which are primarily comprised of antigenic fimbriae and promote adherence to and colonization of the host small intestine (Gaastra & Svennerholm, 1996). To date, more than 20 different CFs have been identified including CFA/1, CS1, CS2, CS4, CS14, CS17, CS5, CS7, CS18, CS20, CS3, CS6, and CS10 to CS12 (Isidean, Riddle, Savarino, & Porter, 2011). Besides enterotoxins and CFs, several plasmid-carried and chromosomally encoded non-classical virulence factors such as ClyA (a haemolysin), LeoA (a cytoplasmic protein), EatA (a serine protease autotransporter), enteroaggregative heat-stable toxin 1 (East-1, a toxin that shares 50% identity with the enterotoxigenic domain of heat-stable toxin), and Tia and TibA (2 outer membrane proteins that mediate adhesion and invasion) have been identified as being involved in the ETEC pathogenicity as well (Del Canto et al., 2011; Rivera et al., 2013; Turner et al., 2006). The detection of these virulence factors is very important to understand the pathogenic characteristics of ETEC strains.

Serotyping is an important tool for the differentiation of ETECs in epidemiological investigations, and of value in identifying certain serotypes. However, serotyping alone is not sufficient to characterize a strain comprehensively. In recent years, various genotyping methods such as pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) have been used to differentiate pathogenic *E. coli* and determine the genetic relationships of strains (Kjelstrup, Arnesen, Granquist, & L'Abée-Lund, 2013; Koo, Kwak, Yoon, & Woo, 2012). PFGE is considered as the gold standard method because of its high level of discrimination, whereas MLST is increasingly being used for pathogen typing owing to the ease of data interpretation via the database comparison. Together, the combination of phenotype and genotypic assays may help to better understand the epidemiological characteristics of ETEC.

With the wide and uncontrolled use of antibiotics in humans and the environment, antimicrobial resistant *E. coli* have been reported from different sources in many countries (Canizalez-Roman, Gonzalez-Nuñez, Vidal, Flores-Villaseñor, & León-Sicairos, 2013; Tan, Lee, & Mahyudin, 2014; Yang et al., 2011). Since antibiotic resistant strains in foods can be transmitted to humans through their consumption and handling, surveillance and monitoring of drug resistant bacteria in food is very important to implement targeted control strategies and select effective drugs for treatment.

As the most commonly present diarrhoeagenic *E. coli* (DEC), ETEC has been detected in different types of foods in China (Liu, Wang, Xie, Sun, & Ji, 2014; Yang et al., 2011). However, the prevalence and molecular characteristics of ETEC in RTE foods have never been systematically investigated. Until now, little information is available on the virulence and genotypic characteristics of ETEC isolated from food samples. The aim of this study was to investigate the occurrence of ETEC in retail RTE foods and to determine the serotypes, virulence factors, antibiotics resistance, and genetic diversity of ETEC isolates.

2. Materials and methods

2.1. Sample collection

From May 2013 to April 2014, a total of 559 RTE foods were collected randomly from open markets and supermarkets in 19 provinces (24 cities) of China. The geographic locations of the sampling sites are presented in Fig. S1. The samples included 192 cooked meats (water cooked pork, water cooked chicken, water cooked duck, and water cooked beef), 43 fried rice, 67 cold vegetable dishes in sauce, 57 cold noodles in sauce, and 200 roast poultry (roast chicken and roast duck). Each sample unit was at least 500 g. The samples were placed in separate sterile plastic bags to prevent cross contamination and immediately transported to the laboratory in a cooler with ice packs and processed within 4–6 h.

2.2. Isolation of E. coli and estimation of most probable number (MPN)

All bacteriological media unless otherwise indicated, were purchased from the Guangdong Huankai Co. Ltd (Guangzhou, China). *E. coli* was isolated from food samples according to the National Food Safety Standards of China (GB/T 4789.6–2003), with some modifications. Briefly, 25 g food sample was placed into a sterile bag containing 225 mL Butterfield's phosphate-buffered water and homogenized at 230 rpm for 2 min using a stomacher. Serial 10-fold dilutions were prepared up to $1{:}10^3$ and 1 mL of serial dilutions (10 (-1), 10 (-2) and 10 (-3)) was inoculated by triplicate in tubes containing 9 mL lactose broth and fermentation tubes. After incubation at 37 °C for 24–48 h, a loopful of suspension from positive cultures (lactose fermentation positive and gas production positive) was streaked onto Chromagar *E. coli* agar plates and incubated at 37 °C for 18–24 h. Subsequently, 2–3 presumptive *E. coli* colonies were selected from each plate and biochemically identified by API 20E (bioMérieux, Beijing, China). The MPN of *E. coli* was calculated reference to the *E. coli* MPN table.

2.3. PCR confirmation of ETEC

All confirmed *E. coli* strains were grown overnight in lactose broth at 37 °C. Genomic DNA was extracted using a commercial Universal DNA Extraction Kit (Sangon Biotech, Shanghai, China) according to the manufacturer's instructions. ETECs were identified by amplifying the *lt* and *st* genes as previously described (Mohammed, 2012). The primer sequences, amplification conditions, and amplicon sizes used in this study are shown in Table 1. All oligonucleotide primers were synthesized by Sangon Biotech. The PCR mixture (total volume 25 μL) contained 1 × DreamTaq™ Green PCR Master Mix (Fermentas, Waltham, MA, USA), 4 μL primers mixtures, and 2 μL DNA template. The PCR was conducted in a Bio-Rad PTC-200 Thermal Cycler (Bio-Rad, Hercules, CA, USA). Genomic DNA of the reference strain ETEC O25:K7 (*lt* + *st*+) and *E. coli* ATCC 25922 were used as positive and negative controls. The amplified products were analysed by electrophoresis on 1.5% agarose gels containing Gold View (SBS Genetech, Beijing, China), and the bands were visualized using an ImageQuant 350 Capture system (GE Healthcare, Waukesha, WI, USA). The isolates that were positive for the *lt* or *st* genes or both were considered as ETEC.

2.4. Detection of CF genes and non-classical virulence genes

Multiplex PCR assays were performed to detect different CF genes as previously described (Nada et al., 2010). The primers used in this study are shown in Table 1. PCR assays were carried out in a 50 mL volume with 2 U DNA Taq polymerase (TaKaRa ExTaq, Tokyo, Japan) in a thermal cycler (PTC-200, Watertown, MA, USA) under the following conditions: initial denaturation at 94 °C for 5 min; 30 cycles each of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min; and a final cycle at 72 °C for 5 min. The amplified PCR products were analysed by gel electrophoresis on 2% agarose gels containing Gold