Prevalence of mobile antimicrobial resistance genes carrying and toxin producing pathogens in retail beef and mutton

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Research article

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Abstract

Background Food safety has always been the global issue. In addition to food poisoning problems caused by toxin-producing strains, the arising of widely spread antimicrobial resistant bacteria have become a major issue that impacts food safety. The purpose of this study is to assess the safety of retail meat in the last link of sales. Results A total number of 134 samples were collected and 674 strains were isolated. There are different bacterial compositions distributed in meat, environmental and human-derived samples. The major pathogens in meat and environmental or human samples are Klebsiella spp. and Staphylococcus spp.. The resistance to amoxicillin + clavulanate (with the resistance rate of 46.78%), tetracycline (44.66%) and erythromycin (32.73%) are major resistant phenotypes of the isolates. According to the whole genome analysis, two K. pneumoniae strains harboring the extended spectrum beta-lactamase genes which are located on mobile elements and two Aeromonas hydrophila isolates carrying mcr-7.1 like genes have been detected. The major toxin genes of Bacillus cereus, and adhesion or invasion related virulence factors were also shared among the isolates. Conclusion There are different pathogens distributed in meat, environment and human source at the final stage of meat consumption. The mobile ARGs are prevalent in strains isolates from meat samples. And toxin-producing strains can be isolated from human source. These factors consist potential risk for public health and need attention.

Introduction

The global spread bacterial resistance poses great threats to public health [1, 2]. The Centers for Disease Control and Prevention (CDC) threat report shows that antibiotic-resistant infections cause at least 2 million diseases and 23,000 deaths in the United States each year [3]. The antimicrobial resistance genes (ARGs) companied with mobile genetic elements (MGEs) can accelerate the spread of antibiotic-resistant bacteria [4, 5]. For instance, extended-spectrum beta-lactamases (ESBLs), firstly reported in 1983, showed a mutation of β-lactamases SHV-1 cause failure of cephalosporin treatment. However, the number of ESBLs have exceeded to more than 200 in 2005. In addition, the ESBLs are usually plasmid mediated, which have been reported origination from more than 30 different counties [6].

Escherichia coli and *Staphylococcus aureus* are the most studied foodborne pathogens [7, 8]. Foodborne pathogenic *E. coli* such as *E. coli* O157 and O104 are commonly found in food poising cases, which cause severe diarrhea [9, 10]. The pathogenic *E. coli* can transmit to humans through consumption of contaminated foods, such as raw meat and raw milk [11, 12]. According to the World Health Organization (WHO)' report, the epidemic of pathogenic *E. coli* caused a loss of US$1.3 billion to Germany's farmers and industries in 2011 [13]. Pathogenic *S. aureus* is notorious for its production of heat-stable toxins. According to the Food and Drug Administration (FDA)' s Bad Bug Book, *Aeromonas hydrophila* and other spp., *Bacillus cereus* and other *Bacillus* spp., *Klebsiella* and *Proteus* have also been suspected of inducing food poisoning or causing acute and chronic gastrointestinal diseases [14]. Additionally, the transmission of ESBLs, methicillin-resistant *Staphylococcus aureus* (MRSA) and *Klebsiella pneumoniae* carbapenemase (KPC) etc. also pose big threat to food safety and public health [15].

Meat shares a large proportion of animal-derived foods which makes it as a major cause of foodborne illness [16]. Pathogenetic bacteria can spread through the food chain - from when the animals are raised, to the day they being slaughtered, till the stage of their sales. There have been a series studies on investigating foodborne pathogens in raw meat, cooked meat or ready-to-eat meat [17-19]. However, the impacts caused by related environment and human beings are often overlooked.

In this study, we collected samples related to retail meat from four different sources: cooked meat, raw meat, environment and human beings. We isolated the strains in different samples to compare the differences in the strain composition. We investigated the antimicrobial resistance phenotypes of the major pathogens, and analyzed the genetic environment of the ARGs in resistant strains. Additionally, we screened the virulence factors in the whole genomes of potential pathogens. In summary, we hope to comprehensively evaluate the safety of meat food industry chain in its terminal segment through the above surveys.

Materials And Methods

Sample collection. In this study, we collected cooked beef and mutton, raw beef and mutton, environmental samples and human samples from local markets in different districts of Beijing to investigating the potential hazards in the last link of meat sales (**Figure 1**). Environmental samples include swabs of chopping board, plate and package. Human samples are swabs of raw meat salespeople's hands, aprons and cleaver. All samples were independently kept in sealed and sterile plastic bags and transported directly to the laboratory for testing within 24 hours after collection.

Bacterial isolation and identification. The isolation process was performed according to the previous protocol [20-22]. Briefly, 2 grams of meat were mixed with 10 mL 0.1% buffered peptone water. After homogenization, 1 mL mixture was transferred into 5 mL BPW and then 1 mL Brain Heart Infusion (BHI) broth and incubated at 32 ^{o}C with 180 rpm for 16 hrs. Then 100 uL suspension was streaked on Columbia agar with 5% sheep blood. Single colonies were transferred into 1 mL BHI broth and incubated at 32 ^{o}C with 180 rpm for another 16 hrs. Bacterial species identification was performed by Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonik GmbH, Bremen, Germany) [23].

Antimicrobial susceptibility tests. The tested antimicrobials include amoxicillin + clavulanate, ceftazidime, ciprofloxacin, ceftiofur, erythromycin, florfenicol, gentamicin, meropenem, polymyxin B, rifampicin, tetracycline, vancomycin. Antimicrobial susceptibility to polymyxin B were assessed by the broth micro-dilution method and the minimum inhibitory concentrations (MICs) for other antimicrobials were determined by an agar dilution method. All operations followed the Clinical and Laboratory Standards Institute's performance standards (CLSI, M100-S29). The *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922 were used as quality control for Gram-positive and negative strains. The statistical significance was carried out by paired t test and determined by GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA).

Bioinformatics analysis. Bacterial genomes were extracted by TIANGEN-tractor (Tiangen biotech, Beijing, China) and sequenced using the Illumina HiSeq ×10 system (Annoroad, Beijing, China). The draft assemblies of the sequences were obtained using CLC Genomics Workbench 9.0 (CLC Bio, Aarhus, Denmark). The draft whole genomes were searched against the ResFinder database and VFDB database using the SRST2 program (version 2-0.1.6) to retrieve all the resistance genes and virulence factors [24]. The contigs carrying ARGs were extract, annotated and searched for the related MGEs according to the ACLAME, ICEberg and Gypsy database [25, 26].

Results

Sample collection and bacterial isolation

During April to July 2018, a total number of 134 samples, containing 37 cooked meat, 53 raw meat, 26 environmental samples and 18 human samples, were collected from 24 markets in four districts of Beijing (**Table 1**). Among 674 isolates, 172 (composed by 26 different genera) were isolated from cooked meat, 330 (26 different genera) were recovered from raw meat, 91 (22 different genera) from environment samples and 81 (15 different genera) from human samples.

Difference in bacterial composition of different samples

Samples from different sources show differences in bacterial composition. For example, the major bacterial species in cooked meat, raw meat, environmental samples and human samples are *Proteus* (41/172, 23.84% of the total isolated strains in cooked meat), *Proteus* (74/330, 22.42%), *Macrococcus* (18/91, 19.78%) and *Staphylococcus* (26/81, 32.10%), respectively. The ten most diverse bacteria in different samples are shown in supplementary **Figure S1**. On the other hand, there are several genus, such as *Aeromonas*, *Bacillus*, *Enterobacter*, *Enterococcus*, *Escherichia*, *Klebsiella*, *Macrococcus*, *Proteus* and *Staphylococcus* are common in different samples (**Figure 2A**).

According to the 2013 CDC report on antibiotic resistance threats and the 2017 WHO list of multidrug-resistant bacteria [3, 27], we select 220 isolates, which belonging to the following genus *Bacillus*, *Enterococcus*, *Staphylococcus*, *Acinetobacter*, *Aeromonas*, *Escherichia* and *Klebsiella*, as the potential foodborne pathogens for further studies. The major pathogenic bacteria are *Klebsiella* and *Escherichia* in the meat samples. However, *Staphylococcus* shows the main threat in environmental and human-derived samples **(Figure 2B)**.

Antimicrobial susceptibility tests of isolated potential pathogenetic strains

To understand the profiles of antimicrobial resistance in the 220 selected strains, all isolates were tested with the antimicrobial which were frequently used in clinical treatment. Generally, isolates mainly show resistance to amoxicillin + clavulanate, tetracycline and erythromycin with the rate of 46.78% (103/220), 44.66% (98/220), 32.73% (72/220), respectively (**Table 2**). It is noteworthy that *Acinetobacter*, *Escherichia* and *Klebsiella* isolates show resistance to ceftazidime with the rate of 37.50% (3/8), 5.26% (2/38), 9.09%

(5/55), respectively. 12.50% (1/8) of the *Acinetobacter* isolates, 5.56% (2/36) of *Aeromona* isolates, 2.63% (1/38) of *Escherichia* isolates and 9.09% (5/55) of *Klebsiella* isolates are resistant to polymyxin B. None of the isolates show resistance to meropenem and vancomycin. Additionally, according to our results, the Gram-positive strains are more resistant to broad-spectrum antimicrobials (P value = 0.02), and the isolates from human samples shows higher resistance rate (P value < 0.01), as indicated in **Table 2**.

Antimicrobial resistance genes and their mobility

According to the results of antimicrobial susceptibility tests, *Acinetobacter*, *Aeromonas*, *Escherichia* and *Klebsiella* isolates showing resistance to ceftazidime and polymyxin B, were subjected to whole genomic sequencing, and their whole genomic sequences were searched against ResFinder to screen ARGs. 40.00% (2/5) of the analyzed *Klebsiella* isolates carry ESBL genes, such as $bla_{\text{OKP-B-10}}$, bla_{SHV28}, $bla_{\text{CTX-M-15}}$ and $bla_{\text{TEM-1B}}$. In the genomes of *Acinetobacter* isolates, the specific ESBL genes $bla_{\text{OXA-130}}$ and $bla_{\text{OXA-51}}$ were detected with rate of 66.67% (2/3). Interestingly, the presence of *mcr-7.1* like gene, leading the resistance to polymyxin B, was found in two *Aeromonas hydrophila* strains (MIC value = 128 μg/mL), both isolated from raw meat. And their sequence identity with the *mcr-7.1* gene (GenBank accession number: MG267386.1) is 81.34% and 77.24%, respectively (**Table S1**).

We screened MGEs and annotated the flanking genetic contexts of the selected ARGs to predict their mobility. According to the results, two *K. pneumoniae* isolates harbor the ESBL genes which are located on mobile elements, as shown in **Figure 3**. The *K. pneumoniae* CNN2-10 is isolated from raw meat. It carries the transposon Tn*2* harboring $bla_{\text{CTX-M-15}}$ and $bla_{\text{TEM-1B}}$, and transposon Tn*2003* harboring $bla_{\text{SHV-28}}$. And the *K. pneumoniae* CYXC3-2 from cooked meat carries the transposon Tn*2003*, which harboring the $bla_{\text{OXA-B-10}}$ gene. The genetic constitutions of the two transposons are siminar with former reports [28, 29].

Prevalence of virulence factors

In our study, bacterial genus, such as *Bacillus*, *Enterococcus*, *Staphylococcus*, *Escherichia* and *Klebsiella* which reported showing various virulence factors, were also selected for whole genomic sequencing. The presence of toxin gene *ces*, encoding the synthesis of cereulide synthetase, were found in two *B. cereus* strains (2/6, 33.33%). Additionally, the virulence factors related to adhesion and invasion were identified in various bacterial species (**Table 3**). For instance, all the *Acinetobacter* isolates carry *ompA*, which is involved in bacterial adherence and invasion. In *Escherichia* isolates, the detection rates of adherence and invasion factors like *fdeC*, *fimH*, *ibeC and ompA* were 33.33% (1/3), 33.33% (1/3), 33.33% (1/3), 66.67% (2/3), respectively. The major adherence and invasion factors in *Klebsiella* isolates are *wabG*, *fimH*, and *mrkD* with the rate of 91.67% (11/12), 66.67% (8/12) and 58.33% (7/12) respectively.

Discussion

Food-borne pathogens play an important role for food poisoning and foodborne infections. Basing on the analysis of the bacterial composition in different sample sources (**Figure 2B**), the main pathogen carried in meat is Enterobacteriaceae, which shows high frequencies of antimicrobial resistance and being host for various ARGs [30-32]. *Staphylococcus* presents as major pathogens in environmental and human samples. It has been reported that *S. aureus* isolates from ready-to-eat foods do not commonly originate from animals but more likely come from food handlers who contaminate foods [33]. Moreover, *B.cereus*, a spore forming foodborne pathogen, was mainly detected in human-derived samples, which may contaminate meat and causing food poisoning [34]. In short, the bacterial compositions show difference in the four sample sources, thus more attention should be paid to the hygienic conditions of operators and the environment to avoid cross-contamination.

According to the antimicrobial susceptibility results, the Gram-positive strains are more resistant to broad-spectrum antimicrobials (*P* value = 0.02), and the isolates from human samples shows higher resistance rate (*P* value < 0.01), as indicated in **Table 2**. We select the isolates which are resistant to ceftazidime (10/137, 7.30%) and polymyxin B (14/137, 10.22%) for further investigation. Two *K. pneumoniae*, isolated form meat samples and harboring mobile ESBL genes, are detected (**Figure 3** and **Table S1**). Additionally, these two *K. pneumoniae* isolates both carry virulence factors related to adhesion and invasion (*wabG*, *fimH* and *mrkD*). *K. pneumoniae*, especially the *K. pneumoniae* producing carbapenemase (KPC), is known as causing various healthcare-associated infections [35, 36]. Studies show that *K. pneumoniae* are prevalent in chicken and pork, these contaminated meats may become an important source of human exposure to *K. pneumoniae* [37]. Therefore, basing on our results, the two *K. pneumoniae* isolates in meat pose a potential risk to the health of consumers. Interestingly, we detect two polymyxin B resistant *A. hydrophila* (2/14, 14.29%, MIC = 128 μg/mL) which harboring the *mcr-7* like genes (sharing the identities with 81.34% and 77.24%). The *mcr-3* and *mcr-5* genes have been widely identified in *Aeromonas* spp. [38, 39]. A study has reported that the amino acid sequence of MCR-7.1 shares 70%, 36% and 69%-81% identifications with MCR-3, MCR-5 and phosphoethanolamine transferase from *Aeromonas* sp., respectively. It is suggested that these *mcr-3*, mcr-5 and *mcr-7.1* may all originate from *Aeromonas* species [40]. Our findings are consistent with this hypothesis. Moreover, many studies have reported that the various *mcr* genes are located in plasmids with mobility [41, 42]. The *mcr-7.1* like genes detected in our study need further study on their location and transferability.

B. Cereus is a major pathogen of food poisoning, mainly caused by its toxins. In this study, all *B. cereus* group isolates show hemolysis. Other than common toxins of *B. cereus* group strains, such as non-haemolytic enterotoxin, haemolysin BL and cytotoxin K1, can cause cell hemolysis, *Bacillus* spp. can also secrete many unknown toxins with hemolytic activity [43]. Cereulide is another major toxin produced by pathogenic *B. cereus*, which can lead to nausea and vomiting, or acute liver failure [44]. Cereulide is a heat-sable lipophilic cyclic dodecadepsipeptide, that makes it survival from the ordinary processing. Cereulide is encoded by cereulide synthetase gene cluster (*ces*), which is located on plasmids and could transfer between *B. cereus* group strains [45]. In this study, we identify two emetic *B. cereus* strains harboring complete *ces* gene cluster, one isolated from raw meat and the other from environment. The *ces*-positive *B.cereus* may contaminate the meat and disseminate through the food chain, which consist

potential hazards to public health. Additionally, the whole genome sequencing analysis shows that *Acientobacter* spp., *Enterococcus* spp., *E. coli* and *K. pneumoniae* isolates in meat samples generally carry virulence factors associated with adhesion and invasion (**Table 3**), which also pose a potential threat to consumers' health.

In summary, there are different pathogens distributed in meat, environment and human source at the final stage of meat consumption. The mobile ARGs carrying pathogens are present in meat. Virulence factors are prevalent in strains isolates from meat samples. And toxin-producing strains can be isolated from human source. These factors could lead to food safety issues and require constant attention.

Declarations

Conflicts of Interest

The authors declare no conflict of interest.

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Tables

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Figures

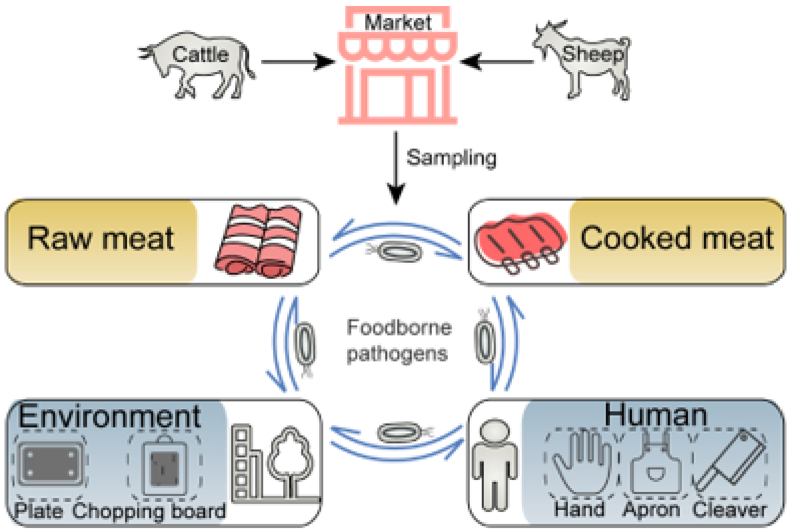

Figure 1

Investigation of foodborne pathogens in retail meat. The raw and cooked meat, environmental samples and human-derived samples were collected from local markets in Beijing, China.

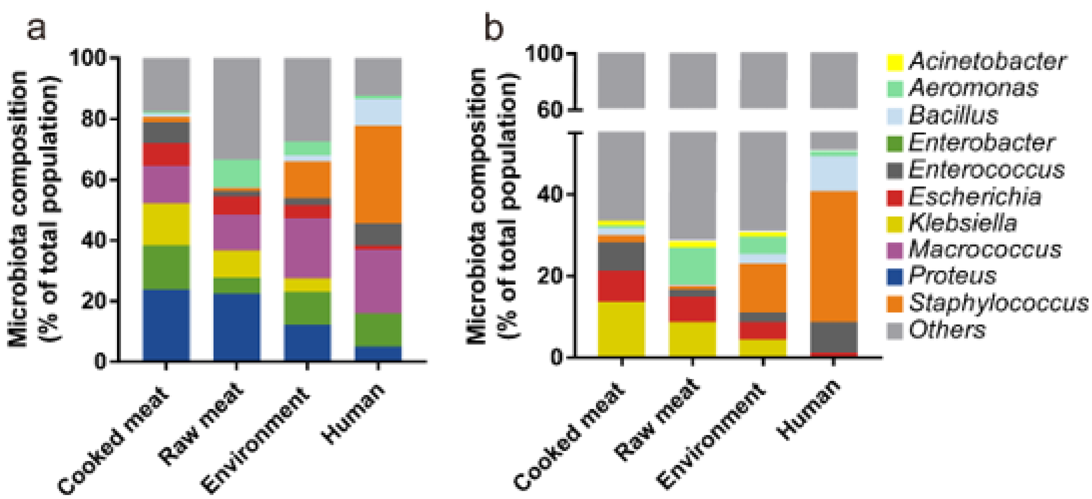

Figure 2

Bacteria composition in samples from four different sources. (a) Proportions of the dominant species in different sample sources. (b) Compositions of major pathogens in different sample sources.

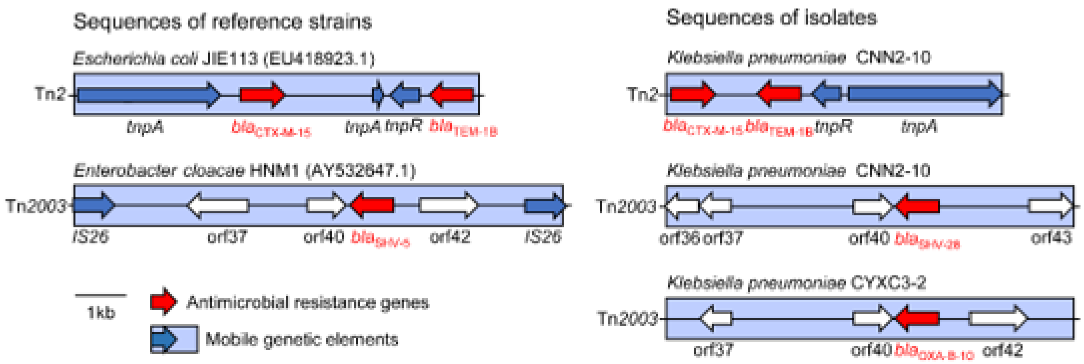

Figure 3

Genetic environment of the ESBL genes in two K. pneumoniae isolates. Two K. pneumoniae isolates harboring the ESBL genes surrounded by mobile elements were identified in this study. The K. pneumoniae CYXC3-2 was isolated from cooked meat, and K. pneumoniae CNN2-10 was isolated from raw meat. The orf36 and orf43 indicate hypothetical proteins; orf37, orf40 and orf42 represent a putative aldolase, DEOR transcriptional regulator and RecF protein, respectively.

Supplementary Files

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