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ESBL- and AmpC-producing *Escherichia coli* from legally and illegally imported meat: Characterization of isolates brought into the EU from third countries

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ABSTRACT

Multiresistant *Enterobacteriaceae* producing extended-spectrum β-lactamases (ESBL) constitute a serious healthcare concern. Contaminated meat and meat products have been suggested as a major transmission route for these strains in the population. In this study, 36 ESBL-/AmpC-producing *Escherichia* (*E.*) *coli* isolates recovered from meat products imported into the EU from third countries (non-EU countries), both legally and illegally, were examined with regard to their antibiotic resistance profiles, virulence-associated genes and their genetic relatedness. The isolates were characterized by antibiotic susceptibility testing, multilocus sequence typing, macrorestriction analysis, microarray analyses and additional PCR assays.

The most prevalent ESBL gene among the isolates was *bla*$_{\text{CTX-M-2}}$ (n = 15), followed by the AmpC β-lactamase gene bla$_{\text{CMY-2}}$ (n = 8). The remaining isolates carried genes belonging to CTX-M groups 8, 1 or 9, or *bla*$_{\text{SHV-12}}$. This distribution differs from the genotypes typically detected in meat-associated isolates of European origin. Most isolates showed additional phenotypic resistances and genes conferring resistance to further antimicrobial agents were detected through microarray analysis. Most commonly observed were resistances to tetracycline, sulfamethoxazole/trimethoprim, and gentamicin. A genotype conferring multidrug resistance to 3 or more classes of antimicrobials could be observed in 33 isolates (91.7%). Most isolates carried at least one gene associated with virulence and one isolate could be identified as an enteropathogenic *E. coli*, indicating a potential risk to consumers' health.

Molecular typing results revealed a genetic variety among the isolates. The most common multilocus sequence types were ST101 and ST117, represented by three isolates each. One isolate belonged to ST131 and three novel sequence types could be identified among three isolates (ST7509, ST7602, ST7845). Group D was the most prevalent phylogenetic group, which was represented by 18 isolates.

Overall, the results of this study show that imported meat products can constitute a source for locally uncommon lineages of multidrug resistant and virulent ESBL-producing *E. coli* and can thereby facilitate their dissemination in Europe.

1. Introduction

Extended-spectrum β-lactamase (ESBL) - producing *Enterobacteriaceae* such as *Escherichia* (*E.*) *coli* are frequently resistant to many classes of antimicrobial agents in addition to β-lactam antibiotics. Consequently, infections with these multidrug-resistant bacteria are correlated with a higher patient mortality as a result of an ineffective initial treatment as well as limited therapy options (Esteve-Palau et al., 2015; Song et al., 2009). In addition, they are associated with considerably higher treatment costs (Esteve-Palau et al., 2015). Food-producing animals are considered a reservoir for *E. coli* causing infections in humans (Founou et al., 2016; Manges and Johnson, 2015). These strains typically spread through the food chain as a result of improper handling or insufficient cooking and can then lead to enteric infections or the colonization of the human gut, posing a risk for subsequent extraintestinal infections such as urinary tract infections (Holmes et al.,

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2016; Vincent et al., 2010).

In light of the massive expansion of international trade, imported food products of animal origin have gained increased attention as a route for the global dissemination of foodborne pathogens (Jansen et al., 2016). Therefore, all products imported into the European Union (EU) must meet certain microbiological criteria laid down in Commision Regulation (EC) 2073/2005 and only imports from selected countries, listed in Regulation (EC) 798/2008, are allowed to enter the EU. However, substantial amounts of products of animal origin also enter the EU illegally through various pathways like traveller luggage or via packages sent by mail and they are typically intended for personal consumption. These products cannot be monitored efficiently and have been discussed as a neglected route of transmission for different enteric viruses and bacterial pathogens such as *Listeria monocytogenes*, verotoxigenic *E. coli*, and *Salmonella* spp. (Nagy et al., 2015; Rodriguez-Lazaro et al., 2015; Schoder et al., 2015).

Multiresistant isolates are of additional concern as many resistance determinants, including ESBL genes, are commonly associated with mobile genetic elements such as plasmids. As a result, they can be easily transferred to other pathogens as well as to commensal bacteria, facilitating both a global spread of antibiotic resistances and the dissemination across different species (Moxon and Paulus, 2016). It has already been demonstrated that illegally imported products of animal origin can harbor multiresistant and virulent strains of *Staphylococcus aureus* that differ from typical livestock- and meat-associated strains circulating in the EU (Müller et al., 2016; Rodríguez-Lázaro et al., 2015). However, to date, very little data is available regarding the characteristics of ESBL-producing *E. coli* entering the EU in this way (Nagy et al., 2015).

In this study, ESBL-/AmpC-producing *E. coli* isolated from meat and meat products imported to Germany from third countries were examined. These products consisted of pork and poultry meat, including illegally imported samples in addition to legal imports. The objective was to characterize the isolates in detail and to compare the results with those of local lineages in order to identify potential risks for the consumer and the community.

2. Methods

2.1. Origin of the isolates

The isolates analyzed in this study were collected in the course of a survey focused on zoonoses and food safety along global supply chains. In brief, samples of meat and meat products imported to Germany from non EU-countries, both legally and illegally, were collected in the years 2014 and 2015 and examined with regard to microbiological food safety criteria and the presence of zoonotic pathogens. The legally imported samples consisted of poultry meat (n = 516) and commercially available pork filets (n = 136) that were cleared via the border inspection post Hamburg Harbour. The illegally imported samples comprised pork products (n = 171) and poultry (n = 126) meat as well as products thereof, which were confiscated from air travel passengers by the competent authorities at the two major German airports Berlin Schönefeld Airport (SXF) and Frankfurt International Airport (FRA). All samples were kept frozen prior to microbiological analyses.

All *E. coli* isolates suspected to be resistant to third generation cephalosporins, based on visible growth on ESBL Brilliance agar (Oxoid, Wesel, Germany), were kindly provided for further analysis in the course of this study. These isolates all originated from different individual samples. An overview of the source and the countries of origin of the ESBL-/AmpC-*E. coli* isolates is given in Table 1.

Table 1
Origin of the 36 *E. coli* isolates examined in this study.

Number of isolates	Country of origin	Legal status	Matrix
29	Brazil	Legal	Poultry meat
3	Chile	Legal	Poultry meat
3	Egypt	Illegal	Poultry meat
1	Chile	Legal	Pork filet

2.2. Phenotypic ESBL-/AmpC-detection and antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed and the results interpreted according to Clinical and Laboratory Standards Institute (CLSI) documents M07-A9 and M100-S24 (CLSI, 2012, 2014).

Screening for ESBL-/AmpC-production was performed by disk diffusion with cefpodoxime (10 μg) and ceftriaxone (30 μg, Oxoid). Subsequently, confirmatory testing of suspected ESBL/AmpC producers was conducted using Micronaut-S β-lactamase VII microtiter plates (Merlin Diagnostika, Bornheim-Hersel, Germany), containing cefepime, ceftazidime, and cefotaxime as single substances and in combination with clavulanic acid to test for ESBL production as well as ceftazidime and cefotaxime in combination with 3-amino-phenyl-borat (APB) to identify AmpC-*E. coli*. The layout further contains meropenem, ertapenem, and cefoxitin. *Klebsiella pneumoniae* ATCC700603 and *E. coli* ATCC25922 served as positive and negative controls, respectively.

Confirmed ESBL-/AmpC-*E. coli* were further tested for additional antimicrobial resistances using Micronaut-S microtiter plates with a test panel for large animals ("Großtiere 2", Merlin Diagnostika), containing ampicillin, amoxicillin/clavulanic acid, cephalothin, ceftiofur, colistin, enrofloxacin, erythromycin, florfenicol, gentamicin, penicillin, spectinomycin, sulfamethoxazole/trimethoprim, tetracycline, tiamulin, tilmicosin and tulathromycin. *E. coli* ATCC25922 was used as quality control strain.

2.3. Species confirmation and multilocus sequence typing

All presumptive ESBL-/AmpC-*E. coli* were analyzed by MALDI-TOF mass spectrometry (Bruker Daltonics, Bremen, Germany) for species confirmation.

Total genomic DNA of the isolates for PCR reactions was extracted from overnight cultures using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany). Multilocus sequence typing (MLST) was performed as described previously (Wirth et al., 2006). Purified amplicons of internal fragments of seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, *recA*) were sequenced on both strands by Eurofins Genomics (Eurofins Genomics, Ebersberg, Germany). Allelic profiles and the corresponding sequence types were assigned using the *E. coli* MLST database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli). For isolates with presumed new sequence types, DNA samples were sent for full genome sequencing (MicrobesNG, Birmingham, UK) and the resulting sequences were uploaded to EnteroBase (http://enterobase.warwick.ac.uk/) for the assignment of sequence types.

2.4. Phylogenetic grouping and macrorestriction analysis

The isolates were assigned to the four major phylogenetic groups by PCR assays targeting internal fragments of *chuA*, *yjaA*, and TSPE4.C2 as described by Clermont et al. (Clermont et al., 2000; Doumith et al., 2012). Genetic variability among the isolates was further examined via DNA macrorestriction analysis with *Xba*I digestion and subsequent pulsed-field gel electrophoresis (PFGE) following the standardized PulseNet protocol (Ribot et al., 2006). The resulting band patterns were evaluated using the BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium) applying the Dice coefficient with 0.5% optimization and 1% position tolerance.