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Short communication

Extraintestinal pathogenic *Escherichia coli* sequence type 131 *H*30-R and *H*30-Rx subclones in retail chicken meat, Italy

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ABSTRACT

Extraintestinal pathogenic *Escherichia coli* sequence type 131 (ST131), typically fluoroquinolone-resistant (FQ-R) and/or extended-spectrum β-lactamase (ESBL)–producing, has emerged globally. Among clinical isolates, ST131, primarily its *H*30-R and *H*30-Rx subclones, accounts for most antimicrobial-resistant *E. coli* and is the dominant *E. coli* strain worldwide. We assessed its prevalence and characteristics among raw chicken meat samples on sale in Palermo, Italy. A collection of 237 fluoroquinolone resistant and ESBL/AmpC producing *E. coli* isolates, which had been isolated from processed retail chicken meat in the period May 2013–April 2015, was analyzed. Established polymerase chain reaction methods were used to define ST131 and its *H*30 subclones, ESBL, AmpC, and plasmid-mediated quinolone resistance (PMQR) determinants. Amplified Fragment Length Polymorphism (AFLP) was performed to assess the relatedness among ST131 isolates. Out of the 237 *E. coli* isolates, 12 isolates belonged to the phylogenetic group B2. Based on the molecular definition of ExPEC, all isolates were attributed with the status of ExPEC. SNP-PCR results confirmed that nine isolates were ST131. SNP-PCR for *H*30-R and *H*30-Rx subclones showed that six and three ExPEC ST131 were positive for *H*30-R and *H*30-Rx, respectively. The results of AFLP showed that, except for four isolates grouped into two clusters which proved to be indistinguishable, the isolates under study were genetically heterogeneous. To the best of our knowledge, this is the first report of *H*30-R and *H*30-Rx subclones in animal food samples. Our findings appear to support the role of food chain in their transmission to humans.

1. Introduction

Extraintestinal pathogenic *Escherichia coli* (ExPEC) sequence type (ST131), belonging to the phylogenetic group B2, has been reported to cause a wide range of extraintestinal infections, including 70–95% of cases of urinary tract infections (UTI), worldwide (Kim, 2012). In 2008, the previously undefined *E. coli* clonal group ST131 was identified in three continents. Today, ST131 is the predominant *E. coli* lineage among ExPEC isolates worldwide (Nicolas-Chanoine et al., 2014).

The ST131isolates commonly harbor a large number of virulence-associated genes (Banerjee and Johnson, 2014; Blanco et al., 2013; Singer, 2015). Moreover, ExPEC ST131 isolates are commonly reported to be fluoroquinolones resistant (FQ-R) and to produce extended-spectrum β-lactamases (ESBLs), such as CTX-M-15 (Banerjee and Johnson, 2014). Recent molecular epidemiologic studies have elucidated the clonal structure of ST131, which includes multiple ST131 subclones with distinctive resistance profiles, including *H*30, *H*30-R, and *H*30-Rx (Banerjee et al., 2013; Johnson et al., 2015; Price et al., 2013). Potential reservoirs of ST131 include food animals and the food chain, but to date isolates of ST131 have been only sporadically detected among commercial animals and food items (Vincent et al., 2010).

The aim of this study was to investigate the prevalence of ExPEC ST131 and its main subclones *H*30-R and *H*30-Rx in retail chicken meat on sale in the area of Palermo, Italy.

2. Materials and methods

A collection of 237 fluoroquinolone resistant and ESBL/AmpC producing *E. coli* isolates, which had been isolated from retail chicken meat sampled in the period May 2013–April 2015 in Palermo, Italy, was included in this study. One hundred sixty-six out of the 237 isolates had been previously described (Ghodousi et al., 2015).

2.1. PCR analysis of phylogenetic groups and virulence genes

E. coli isolates were assigned to one of the four designated phylogenetic group (A, B1, B2, or D) based on the pattern of *chuA*, *yjaA*, and TSPE4.C2 genes presence, as described by Clermont et al. (2000). ExPEC classification of isolates was based on the presence of two or more of the following virulence genes: *pap* (P fimbriae), *sfa* or *foc* (S/F1C fimbriae), *afa* or *dra* (binding, adhesions), *iutA* (aerobactin receptor), and *kpsM II* (group II capsule synthesis) as described (Johnson et al., 2009). *E. coli* RS218

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(*kpsMT II*, *papA*, *papC*, *sfaS*, *hlyD*), *E. coli* V27 (*kpsMT II*, *papA*, *papC*, *iutA*, *focG*), *E. coli* 2H16 (*papC*, *iutA*, *afa*, *hlyD*), and *E. coli* J96 (*papA*, *focG*) were used as positive control strains. *E. coli* MG1655 was used as a negative control in the reactions.

2.2. Antibiotic resistance genotyping

ESBL/AmpC production was screened in all B2 isolates by using the double-disk synergy test and cephalosporin resistance according with the EUCAST guidelines. ESBL genotype was determined by multiplex PCR using published TEM, SHV, CTX-M-1, CTX-M-2 and CTX-M-9 group-specific primers. A plasmid-mediated AmpC β-lactamase genes targeting PCR, including six groups of the ACC, FOX, MOX, DHA, CIT, and EBC groups, respectively, was performed (Dallenne et al., 2010).

All B2 isolates were investigated for the presence of *qnrA*, *qnrB*, *qnrS* and *aac*(6′)-*Ib* genes according with Cattoir et al. (Cattoir et al., 2007).

2.3. PCR-based detection of $bla_{CTX\text{-}M\text{-}15}$

The CTX-M-15-encoding gene $bla_{CTX\text{-}M\text{-}15}$ was detected by PCR using single nucleotide polymorphism (SNP)-specific forward primer 5′-ATAAAACCGGCAGCGGTGG-3′ and universal reverse primer 5′-GAAT TTTGACGATCGGGG-3′ (Johnson et al., 2012). PCR protocol included 10 min of pre-denaturation at 95 °C, subsequently followed by 33 cycles of 30 s at 94 °C, and 30 s at 67 °C and finally one cycle of 7 min at 72 °C post elongation step. The $bla_{CTX\text{-}M\text{-}15}$-specific 483 bp PCR product was detected by agarose gel electrophoresis. *E. coli* strains MVAST131 and JJ1886 were used as negative and positive control strains, respectively (Price et al., 2013).

2.4. Identification of ST131 clone

All group B2 isolates were screened by PCR for the ST131-associated SNPs in *mdh* and *gyrB* (Johnson et al., 2010; Johnson et al., 2014). *E. coli* strains MVAST131, JJ1886 and BUTI 1-2-1 (H17) were used as positive control strains and *E. coli* MG1655 was used as a negative control (Moulin-Schouleur et al., 2007).

2.5. H30 complex subclone detection and detection of H30-R/Rx-specific SNPs

According to the PCR-based screening, ST131 isolates were classified as the *H*30 complex subclone if they contained *H*30-specific polymorphisms in *fimH* (Price et al., 2013). *E. coli* strains MVAST131 and JJ1886 were used as positive control strains.

Two SNPs that differentiate the CTX-M-15-associated subclone (*H*30-Rx) within the *H*30-R subclone from the rest of the *H*30 complex subclone were interrogated using Sanger sequencing. SNP-200 was detected as a C-to-T transition at position 299 of the 460-bp PCR product generated using forward primer 5′-GACACCATGCGTTTTGCTTC-3′ and reverse primer 5′-TCGTACCGG CAACAATTGAC-3′. SNP-264 was detected as a G-to-A transition at position 287 of the 462-bp PCR product generated using forward primer 5′-GTGGCGATTTCACGCTGTT-3′ and reverse primer 5′ TATCCAGCACGTTCCAGGTG 3′. Isolates that tested positive for both SNPs were regarded as members of the *H*30-Rx subclone. *E. coli* strain JJ1886 was used as positive control strain.

2.6. Molecular typing

Amplified Fragment Length Polymorphism (AFLP) was used for molecular typing of ST131 isolates (Kumar et al., 2013). Primers (Eurofins Genomics, Ebersberg, Germany) were dissolved in sterile water. FAM-labeled *Eco*R1 primers were dissolved in 10 mM Tris–HCl pH 8.0. *Eco*RI adapters were constructed from the following primers: *Eco*RI-1 5′-CTCGTAGACTGCGTACC-3′ and *Eco*RI-2 5′-AATTGGTACGCAGTCTAC-3′. *Mse*I adapters were constructed from the following primers: *Mse*I-1 5′-GACGATGAGTCCTGA G-3′ and *Mse*I-2 5′-TACTCAGGACTCAT-3′. For PCR amplification *Eco*RI primer 5′-GACTGCGTACCAATT C-3′ and *Mse*I primer 5′-GATGAGTCC TGAGTAACC-3′ were used. The *Eco*R1 primer contains no selective nucleotide and the *Mse*1 primer contains 2 selective (—CC) nucleotides.

DNA (250 ng) was digested for 2.5 h at 37 °C with 10 U *Eco*RI and 10 U *Mse*I ((New England BioLabs, Ipswich, UK) and 50 ng DNA equivalent was ligated to 0.25 μM *Eco*RI and 2.5 μM *Mse*I adapters with 1 U T4 DNA ligase ((New England BioLabs, Ipswich, UK) for 3 h at 37 °C.

The *Eco*R1 primer (5 μM), *Mse*1 primer (10 μM), 1 × PCR buffer, 1 0.5 mM $MgCl_2$, 0.2 mM dNTPs, and 1 U DynazymeII ((Finnzymes Diagnostics, Thermo Scientific, Wilmington, DE, US) were used to amplify 2.5 ng DNA restriction fragment equivalent with the following cycling protocol: 2 min 94 °C, 13 × (30 s 94 °C; 30 s 65 °C, decreasing 0.7 °C every cycle; 1 min 72 °C) and 10 min 72 °C, 27 × (30 s 94 °C; 30 s 56 °C; 1 min 72 °C) on an Icycler thermocycler (Bio-Rad Laboratories, Hercules, CA, US).

The restriction/ligation product was diluted 20 times in sterile TE buffer and subjected to the PCR with the *Eco*RI-G and *Mse*I-A AFLP primers. PCR fragments were heat-denatured in the presence of Hi-Di™ formamide (Applied Biosystems) and Genescan™ 1200-LIZ size standard (Applied Biosystems) and separated by capillary electrophoresis using a 3130 GeneticAnalyzer (Applied Biosystems). Data were initially analyzed with GeneMapper V4.0 software and further processed in BioNumerics (version 7.1; Applied Maths, Sint-Martens-Latem, Belgium). PCR fragments (80–700 bp) were analyzed with peak detection settings of 0.2% of OD range and 2% of curve range. Pearson similarity matrix combined with UPGMA clustering hierarchy and PCA was applied to analyze data in BioNumerics v. 6.1 software (Applied Maths, Sint-Martens-Latem, Belgium).

3. Results

Out of the 237 *E. coli* isolates, 12 isolates (5.1%) proved to belong to the phylogenetic group B2. Double-disk synergy test results showed that all B2 isolates were putative ESBL producers. Moreover, they proved to be resistant to ciprofloxacin by disk diffusion test according with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (http://www.eucast.org/clinical_breakpoints/).

The complete results of the PCR tests for the antibiotic resistance determinants are shown in Table 1. Based upon the molecular definition, the 12 B2 isolates were all attributed with the status of ExPEC. The most prevalent virulence factors were *kpsMT II* and *iutA* (Table 1). SNP-PCR results confirmed that nine out of the 12 B2 isolates were ST131. These isolates were positive for both ESBL and PMQR determinants. SNP-PCR for *H*30-R and *H*30-Rx subclones showed that six and three ExPEC ST131 were positive for *H*30-R and *H*30-Rx, respectively. The results of AFLP showed that only two pairs of isolates had indistinguishable AFLP profiles (>95% similarity), while the remaining isolates under study were genetically heterogeneous (Fig. 1).

4. Discussion

The prevalence of FQ resistant and/or ESBL producing *E. coli* ST131 is increasing rapidly worldwide (Manges and Johnson, 2012; Mora et al., 2010). The ExPEC isolates from retail poultry meat have been putatively associated with human infections (Kluytmans et al., 2013; Manges and Johnson, 2012) However, only a few studies have investigated the presence of *E. coli* ST131 in food animals. In particular, until now there is no report about detection of *E. coli* ST131 *H*30-R/*H*30-Rx from retail meat.

The high prevalence of FQ resistant and/or ESBL-producing ExPEC ST131 in chicken meat in our experience is concerning. Though a comparison can be misleading because of substantial differences in sampling of food items and laboratory procedures for *E. coli* isolation, previously reported figures are indicative of lower prevalences.

Table 1
Characteristics of 12 isolates of *Escherichia coli* belonging to the B2 phylogroup from retail chicken meat, Palermo, Italy, 2015.

				Virulence genes							Resistance determinants		
Nr.	ExPEC status	ST131	ST131 subclone	*kpsMII*	*papA*	*sfaS*	*focG*	*afa*	*iut*	*papC*	CTX-M	Amp-C	PQMR
43_3	+	+	*H*30-R	+	+	−	−	−	+	−	−	−	*qnrA*
68	+	+	*H*30-R	+	−	−	−	−	+	−	−	CIT-like	*qnrA*
75_2	+	+	*H*30-R	+	−	−	+	−	−	−	Group 1 non CTX-M-15	−	*qnrA*
76	+	+	*H*30-R	+	−	−	+	−	+	−	Group 9	−	*qnrA*
94_2	+	+	*H*30-R	+	+	−	−	−	+	+	−	−	*qnrA*
140	+	+	*H*30-R	+	−	−	−	−	+	−	Group 2		q*nrA*
37_2	+	+	*H*30-Rx	+	−	−	−	−	+	−	15	−	*qnrA*
40_1	+	+	*H*30-Rx	+	−	−	−	−	+	−	15	−	*qnrA*
46_1	+	+	*H*30-Rx	+	−	−	−	−	+	−	−	−	*qnrA*
117	+	−	/	+	−	−	−	−	+	−	−	−	*qnrA*
137	+	−	/	+	−	−	−	−	+	−	−	−	*qnrA*
142	+	−	/	+	−	−	−	−	+	−	−	−	*qnrA*

Vincent et al. (2010) reported a single non-ESBL-producing ST131 isolate from 417 retail chicken samples analyzed. None of the 141 ESBL/AmpC-producing *E. coli* isolates from raw chicken imported into the United Kingdom from South America was identified as ST131 (Dhanji et al., 2010). Similarly, Egea et al. (Egea et al., 2012) did not detect at all the ST131 clone among ESBL-producing *E. coli* isolates from 33 raw retail meat samples. Conversely, *E. coli* ST131 was isolated from seven out of 100 retail chicken meat samples analyzed in Spain: three isolates were resistant to FQ and CTX-M-9 positive; one tested positive for CTX-M-9 and one was resistant to FQ (Mora et al., 2010).

In our study out of nine isolates of ExPEC ST131, six and three, respectively, were attributed to the *H*30-R and *H*30-Rx subclones. To the best of our knowledge, this is the first report of the ExPEC ST131 subclones *H*30-R and *H*30-Rx in retail chicken meat. Of the three *H*30-Rx positive isolates two carried $bla_{\text{CTX-M-15}}$ and one did not. This finding is consistent with literature data showing that some *H*30-Rx *E. coli* isolates do not carry $bla_{\text{CTX-M-15}}$ (Price et al., 2013). A precise evolutionary history of CTX-M-15 acquisition in *H*30-Rx is still unavailable yet, but it has been hypothesized that this subclone might be under stronger antibiotic selective pressure due to its virulence, causing a more frequent use of antimicrobial therapy compared to other *E. coli* lineages (Price et al., 2013). The findings of our study may be tentatively attributed to several factors, such as the initial colonization status of broilers before processing, the degree of fecal contamination of carcasses through slaughtering, and/or poor hygiene during distribution and marketing (Altekruse et al., 2002). However, our results require further confirmation in a larger study.

AFLP showed that, except for two clusters containing two pairs of isolates which proved to be indistinguishable, the isolates under study were genetically heterogeneous. The apparent inconsistencies between genomic fingerprinting and antibacterial drug resistance genetic profiles could be attributable to the high genome plasticity of *E. coli* and the acquisition of virulence genes, resistance determinants and other functions via mobile genetic structures such as plasmids, pathogenicity islands, and other mobile elements (Lanza et al., 2014). Simultaneously, genomic heterogeneity supports the possible involvement of poultry meat in the carriage through the food chain of many different strains. Contamination of meat products may, indeed, occur with *E. coli* from human source during slaughter, processing and distribution, as opposed to the inferred contamination with avian source isolates. However, the heterogeneity of our isolates rules reasonably out an artifactual inflation of the ST131 prevalence due to handling by food workers and possible cross-transmission during the chicken meat processing and marketing steps.

Due to the worldwide increasing demand for poultry meat products, their safety is a critical public health issue. Detecting *H*30-R and *H*30-Rx subclones in retail chicken meat can strengthen the hypothesis of the role of food chain in the acquisition of these pathogenic organisms by humans.

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The authors declare that they have no conflict of interest.

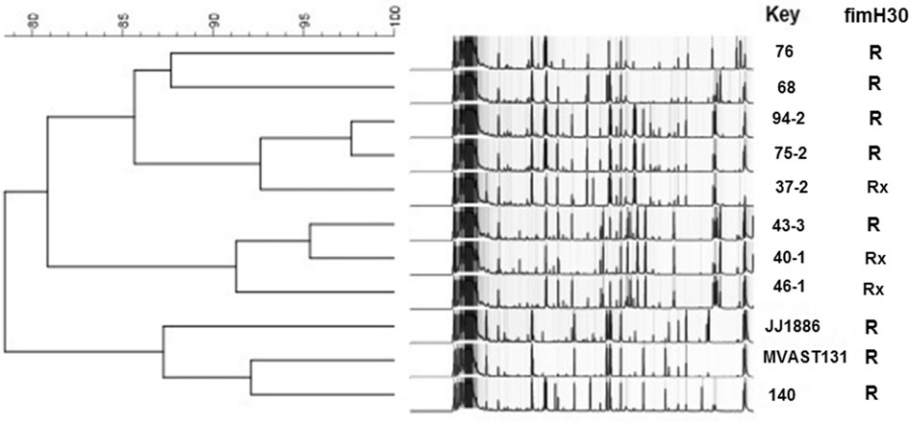

Fig. 1. Amplification fragment length polymorphism (AFLP) analysis of nine *Escherichia coli* isolates from chicken meat belonging to sequence type (ST) 131, Palermo, Italy, 2015.

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