International Journal of Food Microbiology 289 (2019) 162–167

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

ESβL *E. coli* isolated in pig's chain: Genetic analysis associated to the phenotype and biofilm synthesis evaluation

E. Barilli[a], A. Vismarra[a,*], Z. Villa[a], P. Bonilauri[b], C. Bacci[a]

[a] Department of Veterinary Sciences, Food Safety Unit, University of Parma, Italy
[b] Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna – IZSLER - Sezione di Reggio Emilia, Italy

ARTICLE INFO

Keywords:
E. coli
ESβL
Pigs
Pork meat
Antibiotic resistance
Biofilm

ABSTRACT

Resistance to new generation cephalosporins is an important public health problem globally, in terms of economic and social costs, morbidity and mortality. Beta-lactamase enzymes are mainly responsible for the antibiotic resistance of Gram negative bacteria and extended-spectrum-β-lactamases (ESβLs) are one of the major determinants of resistance against oxymino-cephalosporins in *Enterobacteriaceae*. Food-producing animals represent one of the sources of antibiotic resistant bacteria, including pigs.

Here we analysed the presence of *E. coli* resistant to III generation cephalosporins isolated from different matrices collected from intensively bred pigs. A total of 498 *E. coli* were isolated from faeces and carcasses of pigs at slaughterhouse as well as from pork meat and sausages. Among these, 73 were phenotypically confirmed to be ESβL producers. Genetic analysis revealed that all except two harboured at least one of the three selected genes: $bla_{CTX\text{-}M}$, bla_{TEM}, and bla_{SHV}. Furthermore, six of the *E. coli* ESβL isolated from faeces and carcasses swabs, were also able to produce biofilm, highlighting the virulence potential of these strains. The presence of Multi-Drug-Resistance patterns (MDR) recorded by the 73 ESβL *E. coli* was significant (60% of the strains were resistant to more than six antibiotics in MIC test).

Results from the present study show that the transmission of resistant bacteria is possible along the food chain, including production of pork, one the most highly consumed meats around the world. Transmission is possible through the ingestion of raw meat products, and following cross-contamination between raw and cooked foods during preparation. The potential risk for human health demonstrated here, associated with the consumption of pork contaminated with bacterial strains characterized by multidrug resistance patterns, and the ability to produce ESβL and biofilm, is cause for concern. It is imperative to study future control strategies to avoid or limit as much as possible the transmission of these highly pathogenic strains through food consumption and/or contact with the environment.

1. Introduction

Antibiotic resistance (AMR, antimicrobial resistance), the ability of various microorganisms to resist the action of an antibiotic, with consequent ineffectiveness of therapeutic treatment and persistence of the infection has become a major risk to world public health (World Health Organisation, 2014). AMR has been shown to cause not only an increase in economic and social costs, but more importantly is responsible for approximately 25,000 deaths a year in the European Union. It is estimated that the problem could exceed 700,000 cases a year (European Food Safety Authority, 2017). In human medicine, antimicrobial resistance is a widespread problem both within the community and in the hospital environment and can involve common (cystitis, urethritis, skin infections) and/or more complex infections (meningitis, pneumonia, pyelonephritis) (Crivaro et al., 2015).

In human and veterinary medicine, the problem is similar and the use of antibiotics for therapeutic purposes has contributed to the selection and spread of bacterial strains resistant to the majority of available antibiotics. The phenomenon is also complicated by the reduced availability of new antibiotics. Bacteria, under the pressure of antimicrobial use, have developed a series of resistance mechanisms. One of these is the synthesis of hydrolytic enzymes like β-lactamases. β-lactamase enzymes are mainly responsible for antibiotic resistance in Gram negative bacteria and extended-spectrum-β-lactamases (ESβLs) are one of the major determinants of resistance against oxymino-cephalosporins in *Enterobacteriaceae* (Jacoby, 2009). As previously reported, infections due to ESβL-producing pathogens are widely associated with significant morbidity and mortality (Badal et al., 2013).

* Corresponding author at: Dept. of Veterinary Sciences, University of Parma, Via del Taglio 10, 43126, Parma Italy.
E-mail address: alice.vismarra@unipr.it (A. Vismarra).

https://doi.org/10.1016/j.ijfoodmicro.2018.09.012
Received 1 February 2018; Received in revised form 17 July 2018; Accepted 14 September 2018
Available online 17 September 2018

These enzymes are commonly called cephalosporinases, hydrolase penicillins, I–III generation cephalosporins, cephamycins (cefoxitin) and oxyimino-cephalosporins (Jacoby, 2009).

These enzymes have been identified most often in *Escherichia coli*. *E. coli* lodges in the intestine of humans and animals, in the environment, and behaves mainly as a commensal. It can however cause various infections complicated by resistance to antibiotics, in particular infections of the urinary or genital tract (Buelow et al., 2017). The importance of *E. coli* in the spread of antibiotic resistance, in particular mediated by β-lactamases, is related to its diffusion in the environment and to the ability to exchange mobile gene carriers of resistance (plasmids and transposons) with other bacteria.

The β-lactamase encoding genes can be intrinsic or acquired, i.e. by transfer between bacteria. Currently, the most widespread ESβL in Europe is the enzyme CTX-M, followed by the enzymes TEM and SHV, which represent the three main families of ESβL. The CTX-M enzymes, encoded by $bla_{\text{CTX-M}}$, were first identified in 1989 and were termed "cefotaximase-Munich" due to their high efficiency in hydrolysing cefotaxime, compared to ceftazidime, as opposed to the TEM and SHV enzymes (Mathers et al., 2015; Tal et al., 2015). Particularly, the genes of the bla_{SHV} family are for the most part producers of ESβL active against third generation cephalosporins and also monobactam and carbapenem (Liakopoulos et al., 2016).

The expression of ESβL is generally low in many *Enterobacteriaceae*, but inducible when exposed to β-lactam. Many studies have reported a strong and enhanced production of β-lactamases by mutation induced by treatment with III and IV generation cephalosporins. In many bacteria, ESβL enzymes are inducible and can be expressed at high levels by mutation. Overexpression confers resistance to broad-spectrum cephalosporins.

Administration of the same or similar antibiotics to both animals and humans will select the same resistance genes, significantly reducing the lifespan of an antibiotic (van Breda et al., 2017). Animals in this way can act as reservoirs of resistance genes and these can be transmitted directly or indirectly (through food, water) to human pathogens (Davies and Davies, 2010; Marshall and Levy, 2011).

The persistence and spread of antibiotic resistance by *E. coli* is facilitated not only by its diffusion, but also by its considerable resistance in the environment and the ability of numerous strains to form complex communities of bacteria, even of different species, defined biofilms. The presence of biofilms on numerous surfaces concerns the external environment (soil, water systems), the hospital environment and the food chain, where the use of antibiotics, sanitizing and disinfection treatments have favoured the selection of highly resistant microorganisms.

The objectives of the present study included the phenotypic and genetic evaluation of ESβL *E. coli* from the analysis of faeces, carcasses and food from intensively bred pigs. The selected geographical area where we collected the samples is characterized by the highest density of pig farms, slaughterhouses and typical pork meat products (DOP, IGP) in Italy. Moreover, all the confirmed ESβL were subjected to minimal inhibitory concentration (MIC) panels for the evaluation of antibiotic susceptibility. Lastly, the application of a protocol to define their ability to synthesize biofilm was used to further evaluate the virulence patterns of these strains.

2. Materials and methods

2.1. Sample collection

From February 2016 to July 2017, 846 samples from intensive pigs were collected. A total of 200 faeces swabs and 200 carcass sponges were collected from animals coming from five farms located in different provinces of Emilia-Romagna and Lombardy regions (Reggio Emilia, Modena, Parma, Mantova and Cremona). We sampled faeces from different animal groups at the lairage and we follow the same during slaughtering. In this way we randomly collected samples from the same consignment of animals, otherwise a specific relation animal-carcass do not exist. Moreover, 446 pork meat products for human consumption bought at supermarket located in the same geographic zone were part of the sample set.

Faeces samples were harvested in the lairage of the slaughterhouses using sterile swabs and were conserved at 8 °C +/− 2 °C until arrival at the Food Inspection Laboratory of Parma University. Carcass sampling was carried following the UNI EN ISO 17604:2015 method, as described by Commission Regulation EC 2073/2005 (Anonymous, n.d.). Carcasses were selected and sampled with sterile sponges (Sanisponge, VWR chemicals, USA) after slaughter and before the cooling process. Each sponge was placed into a sterile bag and 25 mL of the pre-enrichment broth (Buffer peptone Water, BPW) were added before transport at 8 °C +/− 2 °C to the lab. Pork meat samples (sausages, meat slices, loin, salami dough, cotechino, thighs for ham production) were collected in collaboration with the "Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna".

2.2. Escherichia coli isolation

To each faecal swab, 9 mL of Tryptic Soy Broth (TSB, Biolife, Italia) were added and then incubated at 37 °C for 4 h. Ten microliters of the culture broth were seeded onto Trypton Bile X-Gluc Agar Plates (TBX Agar, Biolife, Italia) and incubated for 18 +/− 24 h at 42 °C

Two hundred-twenty-five mL of BPW were added to each sterile bag containing carcass sponges and incubated overnight at 37 °C. Ten microliters of the culture broth were seeded on Tryptone Bile X-Gluc Agar plates (TBX Agar, Biolife, Italia), according to UNI EN ISO 16649-2:2001, and incubated at 42 °C for 24 h.

E. coli isolation from food samples was done by the Istituto Zooprofilattico Sperimentale of Lombardy and Emilia-Romagna regions following the UNI EN ISO 16649-2:2001.

For the three types of samples, from each plate, a single typical colony, characterized by a blue-green colour, was selected and seeded onto Triptic Soy Agar (TSA, Biolife, Italia). After 24 h of incubation at 37 °C the definitive biochemical identification to the genus level was performed by using the API20E® microsubstrate system (bioMérieux, France).

Positive control *E. coli* ATCC 25922 was used in all the identification phases.

2.3. ESβL E. coli phenotypic evaluation

All *E. coli* isolates were tested for III generation cephalosporin susceptibility through disc diffusion test as defined by European Committee on Antimicrobial Susceptibility Testing (EUCAST) (2015). Cefotaxime (5 μg) and ceftazidime (10 μg) were the antibiotic agents selected. Briefly, confirmed colonies of *E. coli* seeded on TSA plate were used to prepare the inoculum to reach the value of 0.5 McFarland (1.5×10^8 cells/mL), as described by EUCAST (Matuschek et al., 2014). The culture-broth was then seeded uniformly on Mueller Hinton Agar using a sterile swab, antimicrobial discs were added on the plates, and incubated at 37 °C for 18 ± 2 h. To define the resistance (R) or susceptibility (S), we used the zone diameter breakpoints proposed by European Committee on Antimicrobial Susceptibility Testing (EUCAST) (2016): cut-offs for cefotaxime (5 μg) were $R < 17$ mm and $S \geq 20$ mm, while for ceftazidime (10 μg) were $R < 19$ mm and $S \geq 22$ mm.

The isolates with resistance to both cephalosporins tested were phenotypically confirmed with the combination disk test (CDT), as described in European Committee on Antimicrobial Susceptibility Testing (EUCAST) (2015). The CDT was done applying a disc diffusion test using a series of antibiotic agents, including cefotaxime 30 μg, ceftazidime 30 μg, cefotaxime 30 μg with clavulanate 10 μg and ceftazidime 30 μg with clavulanate 10 μg. A MIC was performed on all the strains identified as probable producers of ESßL for the evaluation of antibiotic susceptibility, according to the European Committee on