Detection of Extended-Spectrum Beta-Lactamase producing *Escherichia coli* from mesenteric lymph nodes of wild boars (*Sus scrofa*)

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Abstract

Wild boars (*Sus scrofa*) are increasing in several European countries, including Italy. In areas with intensive animal farming, like the Italian Emilia-Romagna region, they are likely to be exposed to antimicrobial-resistant (AMR) bacteria of livestock origin. In 2017-2018, 108 mesenteric lymph nodes samples were collected from 108 wild boars hunted in Parma province, Emilia-Romagna region, to be tested for ESBL- and carbapenemase-producing *Escherichia coli*. One isolate (WB-21L) out of 108 (0.9%) was phenotypically confirmed as ESBL-producing *E. coli*. The strain WB-21L was tested by PCR for the genes bla_{SHV}, bla_{CTX-M}, bla_{TEM}, bla_{AmpC}, bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP}, bla_{OXA-48}, bla_{SPM}, bla_{BIC}, bla_{SIM}, bla_{DIM}, bla_{GIM}, bla_{AIM}, resulting positive for TEM β-lactamase. Resistance to ampicillin, amoxicillin/clavulanic acid, streptomycin, sulfasomidine, tetracycline and trimethoprim confirmed the multi-resistance nature of the strain WB-21L. Nine *E.coli* isolates showed resistance to meropenem by the Kirby Bauer test but none of them showed Meropenem MIC values indicative of resistance. In conclusion, the present study shows the presence of ESBL *E. coli* in wild boars and the possible risk of transfer to game meat handlers and consumers. Future studies are needed to better evaluate the sources of AMR bacteria in wildlife.

Introduction

Transmission of antimicrobial-resistant (AMR) bacteria or their resistance determinants from food-producing animals to humans has been a public health concern for decades (Moyaert *et al*., 2014). Recently, the role of wild animals in transmitting AMR microorganisms to humans has been investigated in many countries. In particular, wild boar (*Sus scrofa*) populations are increasing in several European countries, including Italy, where they represent the most common among wild ungulates (Carnevali *et al*., 2009). Wild boars are omnivorous and travel large distances for searching food, thus ingesting a large variety of food, including waste (Literak *et al*., 2009). As they often inhabit near humans and livestock animal populations, they can be contaminated by AMR bacteria of human or livestock origin.

Among AMR bacteria, β-lactamase-producing microorganisms are of concern both for human and animal health (Li *et al*., 2007; Poeta *et al*., 2009). β-lactam resistance develops because of different mechanisms, such as inaccessibility of the drugs to their target, target alterations and/or inactivation of the drugs by specific enzymes called β-lactamases. The genes encoding β-lactamases often coexist with other antimicrobial resistance determinants and can be associated with transposons/integrons, thus increasing the potential dissemination of the resistance genes among bacterial species and the emergence of multidrug resistant (MDR) microorganisms (Li *et al*., 2007).

One of the most urgent areas of drug resistance is the evolution of extended-spectrum β-lactamase (ESBL) and carbapenem resistance in Enterobacteriaceae which has spread globally in the last decade (WHO, 2014). Antimicrobial-therapy with cephalosporins (*i.e.* cefotaxime, ceftazidime, ceftriaxone and cefepime) is considered one of the most important treatment options for serious infections due to extraintestinal *Escherichia coli* in humans (Pitout, 2012). The development of resistance against carbapenems (ertapenem, imipenem, meropenem, doripenem) among Enterobacteriaceae is of special concern, because they are often the last line of defence against multi-drug resistant invasive microorganisms belonging to this family (Pitout, 2012).

Among *E. coli*, the production of β-lactamases remains the most important mediator to β-lactam resistance. Classification of β-lactamases is complex and it is based either on molecular classification (Ambler classification) or on functional classification (Bush Jacoby classification) (Ambler, 1980; Bush and Jacoby, 2010). The Ambler classification is based on amino-acid sequences of the enzymes and divides β-lactamases into four classes, namely A, C and D which require serine for β-lactam hydrolysis, and class B metallo-enzymes which require divalent zinc ions for β-lactam hydrolysis. The Bush Jacoby classification uses substrate or inhibitor profiles to divide β-lactamases into three groups: 1) cephalosporinases; 2) serine- β-lactamases; 3) metallo- β-lactamases.

A commonly used definition is that the ESBLs are β-lactamases capable of conferring bacterial resistance to penicillin, first-, second-, third-, and fourth-generation cephalosporins, and aztreonam, but neither to cephamycins or carbapenems, by hydrolysis of these antibiotics, and which are inhibited by β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam and by diazabicyclooctanones (Paterson and Bonomo, 2005; Nordman *et al*., 2012). ESBL-producing *E. coli* have increased constantly during the 2000s, with several reports of nosocomial and community isolates resistant to these antimicrobial classes (Pitout, 2012). The ESBL pandemic in *E. coli* is mostly linked to CTX-M β-lactamases, and especially CTX-M-15 (Pitout, 2012) but other enzymes may be responsible for β-lactams inactivation. For example, during the 1980s and 1990s, the majority of the ESBLs were the SHV or TEM types (Paterson and Bonomo, 2005).

Human invasive *E. coli* isolates resistant to carbapenems have been identified in several EU countries. However, prevalence of resistant isolates was low, ranging from 0.0% to 1% in 2016, and not comparable to

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Key words: *Escherichia coli*, Wild boars, ESBL, Antimicrobial resistance.

Contributions: the authors contributed equally.

Conflict of interests: the authors declare no potential conflict of interests.

Funding: The study was funded by the University of Parma.

Received for publication: 19 July 2018.
Revision received: 15 November 2018.
Accepted for publication: 15 November 2018.

Licensee PAGEPress, Italy
Italian Journal of Food Safety 2018; 7:7707
doi:10.4081/ijfs.2018.7707

resistance among other bacterial species as *Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Acinetobacter baumannii.* In Italy, prevalence of carbapenem-resistant human invasive *E. coli* was 0.3% *in* 2016 (ECDC, 2017). Among livestock animals, pigs were found to be positive for a class B metallo-β-lactamase-producing *E. coli* harbouring the bla_{VIM-1} gene in Germany (Falgenhauer *et al*., 2017) and a class D oxacillinases-producing *E. coli* carrying the $bla_{OXA-181}$ gene in Italy (Pulss *et al*., 2017).

Main purpose of study was the detection of ESBL-producing and carbapenemase-producing *E. coli* in wild boars hunted in northern Italy, Emilia-Romagna region, to assess the likely role of wild animals living in proximity of livestock farms to act as vectors of AMR bacteria. To our knowledge, this is the first Italian study on ESBL- and carbapenemase-producing *E. coli* in wild boars, which aimed at the evaluation of the wildlife/livestock interface in the maintenance of AMR bacteria in an area characterized by intensive livestock farming.

Materials and Methods

Detection of *E. coli* from mesenteric lymph nodes

A total of 108 MLN samples were aseptically collected from the small intestines of 108 wild boars hunted in Parma province, Emilia Romagna Region, northern Italy in 2017-2018. Mesenteric lymph nodes were preferred to faecal samples, because it is still not clear if *E. coli* in faeces are just shedded in short terms, present transient, or cause long term colonization of the gut asymptomatically (Guenther *et al*., 2011). For this survey only animals dead since less than 5 hours were selected. MLN were washed with sterile saline solution and decontaminated using ethilyc alcohol before being placed in sterile containers. The samples were transported to the laboratory at refrigeration conditions. Before being tested, they were cut in small pieces (0.2-0.3 cm) by using sterile scissors. Sample size varied among animals, ranging from 2.5 to 25 g (average tested weight: 21.5 g). One to ten dilutions were realized in Buffered Peptone Water (BPW; Oxoid, Basingstoke, UK) and incubated at 37°C for 18-20 h. A 10 µL loopful of the cultures was streaked onto Tryptone Bile X-Glucuronide (TBX; Biolife Italiana, Milan, Italy) agar plates, incubated aerobically at 44±1°C for 21±3 h. At least three blue-green colonies per sample were selected and plated onto Tryptone Soya Agar (TSA, Oxoid) and Tryptone Soya Broth (TSB, Oxoid), which were incubated at 44±1°C for 21±3 h. Indole production was tested by adding James' reagent to TSB cultures. From TSA plates of indole-positive cultures, one well isolated colony was subjected to species identification with the microsubstrate system Microgen® GN-A (Biogenetics, Padua, Italy).

Testing for ESBL and carbapenemase production

From *Escherichia coli* isolates, a culture of 0.5 Mac Farland's was prepared and seeded onto a Mueller Hinton agar (MHA; Oxoid) plate. The ESBL test was performed by the Kirby-Bauer test following CLSI recommendations (2018a). In addition, carbapenem resistance was evaluated. Disks containing cefotaxime (CTX; 30 µg), ceftazidime (CAZ; 30 µg) and meropenem (MEM; 10 µg) were used and MHA plates were incubated at 35±2°C for 16-18 h. Inhibition diameter zones ≤22 mm for CTX and ≤17 mm for CAZ were considered indicative of ESBL production (CLSI, 2018a). For carbapenems, diameter zones ≤19 mm were considered indicative of non-sensitivity to meropenem. *Escherichia coli* ATCC 25922 was used as quality control microorganism. All the strains which showed a diameter of less than 22 mm for cefotaxime and less than 17 mm for ceftazidime were selected for checking the ESBL production. Phenotypic identification of ESBL-producing isolates was performed by using the ESBL-Confirm Kit (Rosco Diagnostica, Taastrup, Denmark) following the manufacturer's instructions. Briefly, disks containing cefotaxime (30 µg), cefotaxime and clavulanic acid (30 µg; 10 µg), ceftazidime (30 µg) and ceftazidime/clavulanic acid (30 µg;10 µg) were aseptically placed on MHA plates. After incubation at 35±2°C for 18-24 h, ESBL-producing organisms were detected by an at least 5 mm increasing of zone around cefotaxime/clavulanate and/or at least 5 mm around ceftazidime/clavulanate. For carbapenem resistance, isolates showing a diameter zone equal or less than 19 mm for meropenem were tested by the Minimum Inhibitory Concentration (MIC) test following the CLSI guidelines (2018b). Isolates suspicious for carbapenemase-production show Meropenem MIC value ≥4.0 µg/mL.

Testing for β-lactamases genes

To confirm β-lactamase production, the isolates identified by phenotypic tests as ESBL or carbapenemase producers should be tested by PCR for the following genes: bla_{SHV}, bla_{CTX-M}, bla_{TEM}, bla_{AmpC}, bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP}, bla_{OXA-48}, bla_{SPM}, bla_{BIC}, bla_{SIM}, bla_{DIM}, bla_{GIM}, bla_{AIM}. PCR were performed as single reactions to avoid non-specific amplification according to protocols reported in Table 1. Presence or absence of these genes were evaluated without defining the allelic variants.

Testing for antimicrobial- resistance of ESBL *E. coli*

Antimicrobial susceptibility was tested using the Kirby Bauer disc-diffusion method, according to the recommendations of the CLSI (2018a). Mueller-Hinton Agar (Oxoid) and commercial antimicrobial

Table 1. List of PCR detected genes with their amplicon sizes and references to protocols.

Genes	Function	Amplicon Size (bp)	Bibliography
*bla*SHV	Cephalosporinase	747	Monstein *et al.*, 2007
*bla*CTX-M		593	
*bla*TEM		445	
*bla*AmpC - MOX-M		520	Pérez-Pérez and Hanson, 2002
*bla*AmpC - CIT-M		462	
*bla*AmpC - DHA-M		405	
*bla*AmpC - ACC-M		346	
*bla*AmpC - EBC-M		302	
*bla*AmpC -FOX-M		190	
blaKPC	Carbapenemase	*798*	Poirel *et al.*, 2011
*bla*NDM		621	
*bla*VIM		390	
*bla*IMP		232	
*bla*OXA-48		438	
*bla*SPM		271	
*bla*BIC		537	
*bla*SIM		570	
*bla*DIM		699	
*bla*GIM		477	
*bla*AIM		322	

susceptibility discs (HI-Media, Mumbai, India) were used. ESBL-producing *E. coli* were tested against 12 antimicrobials, *i.e.* amikacin (30 µg), ampicillin (10 µg), amoxicillin/clavulanic acid (20µg/10µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), streptomycin (10 µg), sulphasomidine (300 µg), tetracycline (30 µg) and trimethoprim (5 µg). MHA plates were incubated aerobically at 35±2°C for 18-24h. Susceptibility results were categorized as susceptible or resistant according to the CLSI (2018a) recommendations.

Results

One *E. coli* isolate (WB-21L) out of 108 (0.9%) was found to be resistant to cefotaxime and ceftazidime by the Kirby Bauer disc diffusion method. ESBL production was confirmed by the synergy test with clavulanic acid. By PCR, the isolate was positive for the bla_{TEM} gene and negative for the other genes tested. The isolate showed resistant against ampicillin, amoxicillin/clavulanic acid, streptomycin, sulfasomidine, tetracycline and trimethoprim, thus confirm the multi-resistance nature of the WB-21L isolate (R-type:AmcAmpCazCtxSSoTeTmp).

Nine *E. coli* isolates resistant to meropenem were detected by the disc diffusion method, but none of them showed Meropenem MIC values indicative of resistance.

Discussion

To our knowledge, this is the first identification of ESBL-producing *E. coli* in wild boars in Italy. The occurrence of ESBL *E. coli* in wild animals not exposed to antimicrobial agents is of concern, suggesting that the interface livestock/wildlife animals can be effective in maintaining AMR bacteria in the environment. Transmission to wild animals of ESBL bacteria is probably the last step of AMR bacteria environmental pollution.

ESBL isolates harbour resistant genes that code for a variety of β-lactamases. Since β-lactams are among the critically important antibiotics in veterinary medicine, acquired resistance to this large class of antimicrobials is not surprising. The genes encoding these enzymes often coexist with other antimicrobial resistance determinants and can also be associated with transposons/integrons, thus increasing the number of multidrug resistant bacteria as well as dissemination of the resistance determinants among bacterial species (Li *et al*., 2007). For these reasons, ESBL-producing *E. coli* strains may present a phenotype of multi-resistance that included antimicrobial agents of different families (Costa *et al*., 2009). In particular, plasmid-mediated cephalosporinase genes are often associated with coresistance to aminoglycosides, tetracycline and sulphonamides, as the consequence of the colocalization of resistance determinants on the same plasmid (Martinez-Martinez, 2008). This is in accordance with the R-type of the strain WB-21L, which showed resistance to third generation cephalosporins, streptomycin, tetracycline and sulphasomidine.

TEM is considered the archetypical plasmid-encoded β-lactamase (Davies and Davies, 2010). TEM-type beta-lactamases are derivatives of TEM-1, which was first detected in 1965 in an *E. coli* isolate from a patient in Greece, named Temoneira, and of TEM-2 and consist of more than 150 different enzymes (Guenther *et al*., 2011). bla_{TEM}-type enzymes capable of degrading β-lactams have since then disseminated worldwide. Even if the allelic variant of the bla_{TEM} enzyme harboured by the strain WB-21L has not been yet identified, some variants have been previously found in ESBL *E. coli* from wild boars. This is the case of *E. coli* harbouring $bla_{TEM-1,}$ which were identified in faecal samples of wild boars in Portugal (Poeta *et al*., 2009), Check Republic and Slovakia (Literak *et al*., 2009) and *E. coli* carrying $bla_{TEM-52b}$ which were isolated from faeces of wild boars in the Check Republic and Slovakia (Literak *et al*., 2009). In Italy, ESBL *E. coli* harbouring $bla_{TEM-1,}$ $bla_{TEM-24,}$ bla_{TEM-52} and $bla_{TEM-201}$ genes were detected in faeces of pigs and cattle (Stefani *et al*., 2014).

Concerning carbapenemase-producing *E. coli*, the results based on the Kirby Bauer disc diffusion test were not confirmed by Meropenem MIC test.

Conclusions

The global spread, rising incidence, and increased mortality of expanded-spectrum beta-lactamase (ESBL) *E. coli* infections over the past decades have made it one of the biggest threats to human health worldwide (Pitout, 2010). Several studies have demonstrated the occurrence of ESBL *E. coli* in farmed animals, as pigs, cattle and poultry, especially when the animals were treated with third- and fourth-generation cephalosporins (Hammerum *et al*., 2014; Dahms *et al*., 2015). The transfer of ESBL-producing *E. coli* to wild boars could be primarily caused by their habit to visit refuse sites and the proximity of animal farms, eating waste containing resistant *E. coli* (Literak *et al*., 2009). Another source of ESBL *E. coli* in wildlife could be represented by the natural environment, contaminated by AMR bacteria dispersed by different routes, such as livestock manure, manure amended soil, and surface waters polluted with faeces (Kummerer, 2009).

Concerning food-safety, future studies should address whether ESBL *E. coli* can be transmitted by wild boars to the consumers and game meat handlers. The studies of antimicrobial drug resistance in animals living in different natural habitats are warranted to fully understand the importance of wildlife as a source of antimicrobial resistance for humans.

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