E. Coli, K. Pneumoniae and *Providencia Rettgeri ESBLs* producing isolated from pigs in the Veneto region, Italy.

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Abstract

Twelve *Enterobacteriaceae* (n=10 *E. coli*, n=1 *K. pneumoniae* and n=1 *P. rettgeri*) resistant to cefotaxime and/or ceftazidime were isolated from ten sick piglets in the same pig farm in October 2006. All the strains were multi-drug resistant and confirmed as ESBLs producers by synergy tests. PCR and sequencing were carried out to detect the *bla* genes. The *E. coli* isolates and *P.rettgeri* harboured the $bla_{CTX\text{-}M\text{-}1}$; the *K. pneumoniae* isolate were positive for SHV *bla* gene (99% of omology with $bla_{SHV\text{-}28}$). All the isolates but *P. rettgeri* carried a TEM-1 β-lactamase as well.
This study represents the first report of *Enterobacteriaceae* ESBLs-producing other than *E. coli* and *Salmonella* in veterinary microbiology.

Introduction

The spread of resistance to Extended-Spectrum Cephalosporins (ESC) is an issue of growing concern in human medicine (Paterson D. L., 2006). This class of β-lactams as well as fluoroquinolones are antimicrobials widely used in the therapy of complicated infections indeed. Although ESC are not drugs frequently used in veterinary practice, the number of reports recording *E. coli* and *Salmonella enterica* ESC-resistant isolates from animals has recently increased in Europe.
E. coli ESBLs-producing have been isolated from poultry in Spain and Italy (Brinăs et al. 2003,2005, Chiaretto et al. 2006), from cattle in UK (Liebana et al. 2006,) and from companion animals in Italy and Portugal (Carattoli et al. 2005, Costa et al. 2004, Chiaretto et al. 2006). On the other hand, only few recent studies reported the recovery of *E. coli* with ESBL phenotype from pigs (Meunier et al. 2006, Blanc et al. 2006).
The class A ESBLs belong mainly to TEM, SHV and CTX-M family. The CTX-M enzymes, described in late '80 for the first time, have become the most prevalent ESBLs worldwide in humans (Cantón and Coque, 2006). The recovery of *E. coli* and Salmonella carrying $bla_{CTX\text{-}M}$ genes has been also repored from different animal sources (poultry, cattle, pig, pet) in Europe (Weill et al. 2004, Liebana et al. 2006, Meunier et al. 2006, Riaño I., et al., 2006 Carattoli et al. 2005).
The aim of this study was to characterize at a molecular level ESBLs-producing enterobacteria isolated from ten piglets with septicaemia at the same pig farm. This study is the first report of *Enterobacteriaceae* ESBLs-producing other than *E. coli* and *Salmonella* in veterinary microbiology.

Material and Methods

Bacterial Strains
Twelve ESBLs-producing strains (n=10 *E. coli*, n=1 *K. pneumoniae* and n=1 *P. rettgeri*) were isolated from different organs of ten sick pigs (ranging from 30 to 80 days old) after post-mortem examination, in October 2006. The isolates were identified biochemically by routine laboratory procedure and Api20E system (Biomerieux).
Susceptibility tests
The antimicrobial susceptibility was evaluated by disk diffusion with 16 different antimicrobial drugs (Becton Dickinson Microbiology Systems Cockeysville, MD, USA) on Mueller-Hinton (MH) agar, according to the recommendations of the CLSI (formely NCCLS). The following antimicrobials were

tested: colistin (CL 10 µg), trimethoprim-sulfamethoxazole (SXT 23,75+ 1,25 µg), kanamycin (KAN 30 µg), gentamicin (GEN 10 µg), cefotaxime (CTX 30 µg), amoxicillin-clavulanic acid (AMC 30 µg), nalidixic acid (NAL 30 µg), tetracycline (TET 30 µg), ampicillin (AMP 10 µg), streptomycin (STR 10 µg), chloramphenicol (CHL 30 µg), spectinomycin (SPT 100 µg), enrofloxacin (ENO 5 µg), sulfisoxazole (G 25 mg), aminosidin (AN 60 µg), apramycin (APRA 15 µg). The strains were also tested against three extra β-lactams: ceftiofur (30 µg), cefoxitin (FOX 30 µg), imipenem (IMP 30 µg)). The ESBL phenotype was assayed according to CLSI tests using ceftazidime and cefotaxime (30 µg) and the same cephalosporins plus clavulanic acid (10 µg). The enhancement of the oxymino-β-lactams inhibition zone (≥ 5 mm) caused by clavulanate revealed the ESBL-producing strains. *E. coli* ATCC25922 and *K. pneumoniae* ATCC 700603 were used as quality control strains for the ESBL phenotype screening tests.

Genetic characterization of *bla* genes.

Total DNA was extracted with InstaGene matrix kit (Bio-Rad) in accordance with the manufacturer's recommendation. The detection of *bla*TEM, *bla*SHV, *bla*CTX-M genes was carried out by PCR assays.The primers used were either adapted from those previously published (Liebana et al. 2004, Weill et al. 2004) or designed by using computer analysis of available β-lactamase sequences (GenBank) (Table 2).

Table 2: Primers and PCR conditions used in PCR analysis

PCR target	Primer	Sequence (5'-3')	T_a(°C)	PCR products	Reference
bla_{TEM}	TEM-F	TCGTGTCGCCCTTATTCCCTTTTT	60	425 pb	Liebana 2004
	TEM-R	GCGGTTAGCTCCTTCGGTCCTC			
	TEM-B-F	AGTCACAGAAAAGCATCTT	52	576 pb	This study*
	TEM-B-R	GAGTAAACTTGGTCTGACAG			
bla_{SHV}	SHV-F	CGGCCCCGCAGGATTGACT	60	409/849 pb	Liebana 2004
	SHV-Fmod**	GATGTATTGTGGTTATGCGTT			
	SHV-R	TCCCGGCGATTTGCTGATTTC			
bla_{CTX-M}	CTX-M-F	C(A/G)ATGTGCAG(C/T)ACCAGTAA	53	540 pb	Weill 2004
	CTX-M-R	CGC(A/G)ATATC(A/G)TTGGTGGTG			
bla_{CTXM} Group1	CTX-M-F-1	ATGGTTAAAAAATCACTGC	53	886 pb	This study***
	CTX-M-R-1	CGTTTCCGCTATTACAA			

*primers TEM-B-F position: 315-333, TEM-B-R position: 862-881; EMBL accession number AY529705.
**primers SHV-Fmod position: 185-205; EMBL accession number AF124984.
***primer CTX-M gruppo1, CTX-M-F position: 63-81, CTX-M-R position 871-887; EMBL accession number X92506

Sequencing

The purified PCR fragments were sequenced on both strands with the "BigDye terminator v3.1 cycle sequencing" kit (Applied Biosystems) using the same set of primers as for the PCRs (Table 1). Sequence analysis was performed on an 3100-Avant Genetic Analyzer (Applied Biosystems) and analysed using the software SeqScape v2.1.1. The obtained nucleotide sequences and the derived amino acid sequences were compared with those previously described from the GeneBank database www.ncbi.nlm.nih.gov and www.lahey.org/studies/webt.html, respectively.

Results

Ten *E. coli*, one strain of *K. pneumoniae* and one strain of *P. rettgeri* were identified by biochemical tests. All the strains showed a multi-drug resistant phenotype (summarized in table 1) and were confirmed as ESBLs producers by synergy tests. All the *E. coli* strains and the *P. rettgeri* isolate carried a CTX-M enzyme classified as CTX-M-1 by DNA sequencing.

The *K. pneumoniae* isolate was positive to a bla_{SHV} gene, the sequence analysis of the amplicon revealed an omology of 99 % with the bla_{SHV-28} gene. All the isolates but the strain of *P. rettgeri* possessed the β-lactamase TEM-1. The results of genetic characterization are shown in table 1.

Discussion

The CTX-M β –lactamases have become the most widely spread ESBLs worldwide (Cantón and Coque, 2006). In this study, we found multi-drug resistant strains (*E. coli* and *P. rettgeri*) positive for the CTX-M-1 β –lactamase. Among the CTX-M enzymes, CTX-M group1 are recognized mainly in Europe. In particular, the CTX-M-1 β–lactamase is the most prevalent variant reported in human *E. coli* and has been already reported in animals in Italy (Mugnaioli et al. 2006, Carattoli et al. 2005). Furthermore, we found that in the same animal a strain of *Klebsiella pneumoniae* SHV positive and a strain of *E. coli* CTX-M –1 producing coexisted. This study represents the first report of *Enterobacteriaceae* ESBLs-producing other than *E. coli* and *Salmonella* in veterinary microbiology

The strains were isolated from piglets coming from different areas in the same pig farm and we reported also the isolation of two different species (*E. coli* and *P. rettgeri*) carrying CTX-M-1 from the same animal. It is worth noting that the dissemination of CTX-M genes can follow different ways (Cantón and Coque, 2006). In the farm under study, this could be due to the presence of a specific clone and/or mobile genetic elements. Further molecular investigations and monitoring will be needed to ascertain the mechanism/s of CTX-M-1 positive *E. coli* spreading and check the efficacy of the control actions adopted.

Conclusion

The antibiotic resistance is a issue of growing concern in human and veterinary medicine. In particular, antimicrobials such as cephalosporins and fluoroquinolones represent major therapeutical options in complicated human infections. The recovery of ESBLs- producing *Enterobacteriaecae* animal-associated demands surveillance actions focused on cephalosporins resistance to understand the interplay among animals and humans related to the diffusion of cephalosporin resistance genetic determinants. On the other hand, the prudent use of antimicrobials remains the first action to preserve their effectiveness.

Table 1: ESBLs- producing strains isolated from sick piglets (Dicember 2006)

Strain	Species	Resistance	β-lactamases
3593	*E. coli*	SXT-KAN-GM-CTX-AMC-TET-AMP-STR-CHL-SPT-G-AN-EFT	CTX-M-1, TEM-1
3595	*E. coli*	SXT-KAN-GM-CTX-AMC-TET-AMP-STR-G-AN-APRA-EFT	CTX-M-1, TEM-1
3616	*E. coli*	SXT-KAN-GM-CTX-AMC-TET-AMP-STR-G-AN-EFT	CTX-M-1, TEM-1
3620	*E. coli*	SXT-KAN-GM-CTX-AMC-TET-AMP-STR-CHL-SPT-G-AN-EFT	CTX-M-1, TEM-1
3622	*E. coli*	SXT-KAN-CTX-AMC-NAL-TET-AMP-STR-CHL-G-AN-EFT	CTX-M-1, TEM-1
3623	*E. coli*	SXT-KAN-CTX-AMC-CTX-NAL-TET-AMP-STR-CHL-G-AN-EFT	CTX-M-1, TEM-1
3623	*K. pneumoniae*	SXT-KAN-CTX-AMC-NAL-TET-AMP-CHL-ENO-G-EFT	bla_{SHV}, TEM-1
3696	*E. coli*	SXT-KAN-GM-CTX-AMC-NAL-TET-AMP-STR-G-AN-EFT	CTX-M-1, TEM-1
3857	*E. coli*	SXT-KAN-GM-CTX-AMC-NAL-TET-AMP-SPT-CHL-ENO-G-AN-EFT	CTX-M-1, TEM-1
3858	*E. coli*	SXT-KAN-GM-CTX-AMC-NAL-TET-AMP-SPT-CHL-ENO-G-AN-APRA	CTX-M-1, TEM-1
3859	*E. coli*	SXT-KAN-GM-CTX-AMC-TET-AMP-STR-C-SPT-G-AN-EFT	CTX-M-1, TEM-1
3859	*P. rettgeri*	CL-SXT-KAN-CTX-AMC-TET-AMP-STR-C-SPT-G-AN-APRA-EFT	CTX-M-1

CL: colistin, SXT: trimethoprim-sulfamethoxazole, KAN: kanamycin, GEN: gentamicin, CTX: cefotaxime, AMC: amoxicillin-clavulanic acid, NAL nalidixic acid, TET tetracycline, AMP ampicillin,

STR streptomycin, CHL: chloramphenicol, SPT spectinomycin, ENO: enrofloxacin, G: sulfisoxazole, AN: aminosidin, APRA: apramycin, EFT: ceftiofur.

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