Antibiotic resistant *Enterobacteriaceae* from Portuguese piggeries

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ABSTRACT

Background: Antibiotic (AB) overuse in animals, food chain, and trade seem to contribute to the spread of antibiotic resistant (AR) bacteria outside hospitals. Data about AR occurrence among food animals is scarce and limited to specific countries. **Objectives:** To evaluate the occurrence of AR *Enterobacteriaceae* in Portuguese piggeries. **Methods:** We analyzed 57 samples (fresh/dry faeces, nasal/hide, drinking/waste water, feed, air, powder, surfaces, xerume) from 5 Portuguese piggeries located in different regions (2006-07). After enrichment, samples were plated on MacConkey agar with/without AB. Isolates presumptively identified as *Enterobacteriaceae* by standard biochemical profiles were selected for further studies. AB susceptibility (22 ABs) was performed by standard CLSI methods. Presence of extended spectrum β-lactamases (ESBL, conferring resistance to third-generation cephalosporins) was searched by double-disc synergy test. Genes encoding resistance to quinolones (*qnrs*, *qepA*) were searched by PCR. **Results:** We identified 191 *Enterobacteriaceae*, the occurrence of resistant isolates being similar among piggeries. Isolates were commonly resistant to tetracyclines (84%), streptomycin (75%), sulfonamides (72%), and trimethoprim (70%), and also to chloramphenicol (25%), nalidixic acid (16%), other aminoglycosides (<12%) and ciprofloxacin (2%). Among β-lactams, resistance to cefotaxime was frequent (16%). ESBLs were detected among 14% of isolates (23 *E. coli*, 2 *Proteus vulgaris*, 1 *Serratia marcescens*, 1 *Citrobacter freundii*), being consistently recovered from two piggeries. *qnrS* was found in one ESBL-producing *E. coli*. *qnrA*, *qnrB*, and *qepA* were absent among ESBL producers. **Conclusions:** *Enterobacteriaceae* resistant to ABs used in clinical practice are frequently recovered from Portuguese piggeries. The potential dissemination to humans highlights the need for public health efforts to implement surveillance, epidemiologic, environmental health, and policy-making components.

INTRODUCTION

- **Antibiotic overuse in animals could create an important reservoir of antimicrobial resistant bacteria and genes that can spread to humans through the food supply (1, 2).**
- **Some antimicrobial agents used in veterinary and human medicine belong to the same antibiotic families and hence selective pressures exercised in food producing animals environments might contribute for the selection and dissemination of similar resistance genes (3, 4).**
- **To ensure future effectiveness of antimicrobials in human medicine, WHO is developing international consensus guidelines for reducing the use of critically important antimicrobials in food animals (1). Other control measures to reduce the exposure are also being proposed by EFSA (5).**
- **In a previous survey performed in *Enterobacteriaceae* isolates between 1998 and 2004 from faeces of Portuguese swine, dissemination of genes and epidemic plasmids coding for antibiotic resistance (mainly beta-lactams, aminoglycosides, sulfonamides, trimethoprim and quinolones) were reported (6).**
- **Regular data about antibiotic resistance occurrence among food animals and their production environment is scarce and limited to specific countries. Continuous surveillance in a bigger scale, comprising food-producing farms from diverse geographic areas, is imperative for better control of antibiotic resistance.**

OBJECTIVES

- **To evaluate the occurrence of antibiotic resistant *Enterobacteriaceae* in Portuguese piggeries.**
- **To investigate the presence of extended-spectrum beta-lactamase (ESBL) enzymes among *Enterobacteriaceae* recovered from Portuguese piggeries.**
- **To evaluate the occurrence of genes coding for resistance to quinolones among ESBL-producing *Enterobacteriaceae*.**

MATERIAL AND METHODS

Bacterial isolates. **We analyzed 57 samples from 5 intensive-production piggeries (Pg) located in the North (n=18, Pg A), Centre (n=28, Pg C and Pg E), and South (n=11, Pg B) regions of Portugal, recovered between April 2006 and December 2007. Different types of samples were analyzed: fresh/dry faeces, nasal/hide, drinking/waste water, feed, air, powder, surfaces, and xerume. After pre-enrichment in buffered peptone water for 18 h at 37ºC, samples were plated (0,2 mL) on MacConkey agar with and without ceftazidime (1 mg/L), cefotaxime (1 mg/L), tetracyclines (6 mg/L) or sulfonamides (256 mg/L). Presumptive *Enterobacteriaceae* were selected for further studies and identified by using API ID32GN galleries (bioMérieux, Marcy l'Étoile, France).**

Antimicrobial susceptibility testing **to beta-lactam and non-beta-lactam antibiotics was determined by the standard disk diffusion method following CLSI guidelines (7). The antibiotics tested were the following: amoxicillin-clavulanic acid, ceftazidime, cefotaxime, cefepime, cefoxitin, aztreonam, imipenem, ciprofloxacin, nalidixic acid, tetracyclines, chloramphenicol, sulfonamides, trimethoprim, streptomycin, spectinomycin, kanamycin, gentamycin, amikacin, tobramycin, apramycin, neomicin and netilmicin. All intermediate-susceptible isolates were considered as non-susceptible.**

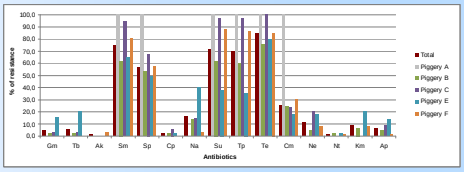
Expression of ESBL **was screened by the standard double disk synergy test using Mueller-Hinton agar plates with and without cloxacillin (250 mg/L) (8, 9).**

Genes encoding resistance to quinolones, namely qnrA, qnrB, qnrS and the recently described qepA gene**, were searched by PCR using primers and amplification conditions previously described (10, 11) (Table 1).**

Table 1. Primer sequences and PCR conditions used in this study

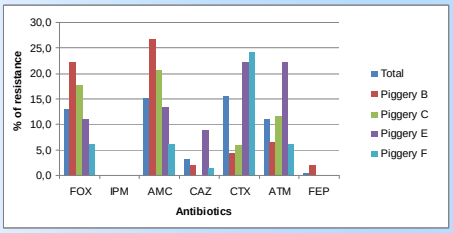
Primer	Sequence (5'-3')	Gene	Size (bp)	Amplification Conditions	[MgCl2]	Reference
qnrAm-F qnrAm-R	AGA GGA TTT CTC ACG CCA GG TGC CAG GCA CAG ATC TTG AC	qnrA	580	1 cycle of 10 min at 94ºC; 35 cycles of 1 min at 94 ºC, 1 min at 53ºC, 1 min at 72ºC; 1 cycle of 10 min at 72 ºC	1,5	10, 11
qnrBm-F qnrBm-R	GGM ATH GAA ATT CGC CAC TG TTT GCY GYY CGC CAG TCG AA	qnrB	264	1 cycle of 10 min at 94ºC; 35 cycles of 1 min at 94 ºC, 1 min at 57ºC, 1 min at 72ºC; 1 cycle of 10 min at 72 ºC	1,5	10, 11
qnrSm-F qnrSm-R	GCA AGT TCA TTG AAC AGG GT TCT AAA CCG TCG AGT TCG GCG	qnrS	428	1 cycle of 10 min at 94ºC; 35 cycles of 1 min at 94 ºC, 1min at 52ºC, 1 min at 72ºC; 1 cycle of 10 min at 72 ºC	1,5	10, 11
qepA-F qepA-R	CGT GTT GCT GGA GTT CTT C CTG CAG GTA CTG CGT CAT G	qepA	403	1 cycle of 10 min at 94ºC; 35 cycles of 1 min at 94 ºC, 1 min at 57ºC, 1 min at 72ºC; 1 cycle of 10 min at 72 ºC.	1,7	10, 11

Fig. 1. Resistance patterns to non-beta-lactam antibiotics among *Enterobacteriaceae* recovered from different Portuguese piggeries



Gm, gentamycin; Tb, tobramycin; Ak, amikacin; Sm, streptomycin; Sp, spectinomycin; Cp, ciprofloxacin; Na, nalidixic acid; Su, sulfonamides; Tp, trimethoprim, Te, tetracycline; Cm, chloramphenicol; Ne, neomycin; Nt, netilmicin; Km, kanamycin; Ap,apramycin.

Fig. 2. Resistance patterns to beta-lactam antibiotics among *Enterobacteriaceae* recovered from different Portuguese piggeries*



(*) The unique isolate from Piggery A was susceptible to all beta-lactams. FOX, cefoxitin; IPM, imipenem; AMC, amoxicillin/clavulanic acid; CAZ, ceftadizime; CTX, cefotaxime; ATM, aztreonam; FEP, cefepime

RESULTS

➢ ***Epidemiological background***

- A total of 191 *Enterobacteriaceae* representing different colony morphotypes and antibiotic susceptibility patterns were obtained from fresh/dry faeces (n=47), nasal/hide (n=10), drinking/waste water (n=68), feed (n=43), air (n=12), powder (n=3), surfaces (n=1), and xerume (n=4) samples.

➢ ***Resistance patterns to non-beta-lactam antibiotics among Enterobacteriaceae from different piggeries***

- Similar resistance patterns to non-beta-lactam antibiotics were observed among isolates recovered from all piggeries. Distribution of resistance patterns to non-beta-lactam antibiotics among *Enterobacteriaceae* from each piggery studied is shown in Figure 1.
- Isolates were mostly non-susceptible (intermediate or resistant) to tetracyclines (84%), streptomycin (75%), sulfonamides (72%), and trimethoprim (70%).
- Non-susceptibility patterns to chloramphenicol (25%), nalidixic acid (16%), other aminoglycosides (<12%) and ciprofloxacin (2%) were more rarely observed.

➢ ***Resistance patterns to beta-lactam antibiotics among Enterobacteriaceae from different piggeries***

- Resistance rates to beta-lactam antibiotics greatly varied among isolates from each piggery evaluated (Figure 2).
- The unique *Enterobacteriaceae* recovered from Pg A was susceptible to all beta-lactam antibiotics. Among Pg B and Pg C, isolates were mostly resistant to amoxicillin-clavulanic acid and cefoxitin (27% and 22% versus 21% and 18%, respectively), indicating a possible high occurrence of AmpC-producing *Enterobacteriaceae*.
- Resistance to cefotaxime was frequently observed in *Enterobacteriaeae* recovered from Pg E and Pg F (22%, 10/45 and 24%, 16/66, respectively).
- Overall, resistance to cefotaxime was the most frequently observed (16%, 30/191) when analysing resistance rates obtained for beta-lactams tested.
- Resistance to cefepime was very low (0,5%) and all isolates were susceptible to imipenem.

➢ ***Occurrence of ESBL phenotypes***

- ESBL expression was observed in 14% (27/191) of the isolates included in this study and identified as *E. coli* (n=23), *P. vulgaris* (n=2), *S. marcescens* (n=1), and *C. freundii* (n=1).
- ESBL-producing *Enterobacteriaceae* were consistently recovered from two piggeries, Pg E and Pg F, localized in the Centre and North regions of Portugal, respectively, and showed a clear spread in these piggeries environment, being detected in different samples: faeces (n=9), hide (n=2), feed (n=8), drinking water (n=4) and wastewater (n=4) samples.
- The majority of the isolates presenting an ESBL phenotype were susceptible to ceftazidime and resistant to cefotaxime, suggesting the dissemination of CTX-M enzymes in Portuguese piggeries.

➢ ***Distribution of multidrug resistant Enterobacteriaceae among different piggeries' samples analysed***

- Multidrug resistant *Enterobacteriaceae* (resistance to 2 or more antibiotics tested) were frequently recovered from xerume (100%, 4/4), drinking/waste water (71%, 48/68), nasal/hide (100%, 10/10), powder (100%, 3/3), and fresh faeces (97%, 33/34) samples.

➢ ***Occurrence of genes coding for resistance to quinolones among ESBL-producing Enterobacteriaceae***

- *qnrS* was found in one ESBL-producing *E. coli* recovered from a feed sample from Piggery E.
- *qnrA*, *qnrB*, and *qepA* were absent among all ESBL-producing *Enterobacteriaceae*.

CONCLUSIONS

- ➢ ***Enterobacteriaceae* resistant to antibiotics used in clinical practice are frequently recovered from pigs and piggeries environment in Portugal.**
- ➢ **We describe a high occurrence of ESBL-producing *Enterobacteriaceae* in Portuguese piggeries. This finding is worrisome as these enzymes confer resistance to cephalosporins of third- and fourth-generation, relevant for human infections treatment.**
- ➢ **Presence of acquired genes conferring resistance to fluoroquinolones suggests the emergence of plasmid-mediated quinolone resistance in *Enterobacteriaceae* from Portuguese piggeries and a possible animal reservoir for the *qnr* genes.**
- ➢ **Our findings are worrisome as transmission of antibiotic resistance to humans via pork products and throughout contamination of environment is likely to occur, compromising several relevant antibiotics for treatment of human infections.**

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