

ANTIBIOTIC RESISTANCE IN ENTEROBACTERIACEAE ISOLATED FROM PORTUGUESE DELI MEATS

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ABSTRACT

This study aimed to identify the presence of β -lactam-resistant bacteria in different types of Portuguese deli meats. The numbers of ampicillin resistant bacteria varied from negative in 25 g to 1.0×10^8 colony-forming units/g. Within 78 randomly selected β -lactam-resistant bacteria, 24 different resistant phenotypes were found and 35.9% were multidrug resistant (MDR). The majority (87.2%) of the isolates identified belonged to the Enterobacteriaceae family. The presence of the bla_{TEM} gene was detected in 23 out of 67 isolates (34.3%) and 16 of them presented MDR phenotypes. Four Klebsiella oxytoca isolates (6%) harbored a gene for the CTX-M/OXY-type enzyme. The direct sequencing of their purified amplicons confirmed the presence of three types of bla_{OXY} genes (bla_{OXY-1}, bla_{OXY-2} and bla_{OXY-5}). These results suggest that without good hygienic practices, deli meats may act as a vehicle of transfer of β -lactam-resistant bacteria to the gastrointestinal tract of consumers.

PRACTICAL APPLICATIONS

In the current regulation on microbiological criteria for foodstuffs the test for Enterobacteriaceae is used as indicator of hygiene and contamination after processing. Nevertheless, the major concern for public health related to the presence of these bacteria in food is because of the emerging antibiotic resistance in this family, namely to b-lactams. Consequently, the ever-increasing consumption of ready-to-eat (RTE) food, can contribute to the spread of these resistant strains. In the present study different types of deli meats, sold at retail outlets in Portugal, presented levels of b-lactam-resistant Enterobacteriaceae that suggested the lack of strict hygiene control measures in the food chain. Without good hygienic practices, the dissemination in the community of antibiotic resistance genes may assume serious proportions.

INTRODUCTION

Antimicrobial agents are widely used to prevent and to treat disease among food-producing animals and in some cases to improve feed utilization and growth rate (IFT 2006). Although providing for the health of food-producing animals, these antimicrobial agents belong to the same antibiotic families used in human medicine and consequently similar resistance genes might emerge because of selective pressures applied in diverse environments (Aarestrup 1999; IFT 2006; Machado *et al.* 2008).

The emerging antibiotic resistance in the Enterobacteriaceae family is a significant problem that requires immediate attention. The increasing prevalence of antibiotic resistant Enterobacteriaceae, not only pathogenic but also commensal (nonclinical) bacteria, suggests that the community can act as a reservoir and that food can contribute to the spread of these resistant strains (Mesa *et al.* 2006). The most common mechanism of resistance among Enterobacteriaceae is the production of b-lactamases, chromosomally or plasmid encoded, which inactivate certain b-lactam antibiotics by hydrolyzing the b-lactam ring (Slama 2008; Smet *et al.* 2008). The persistent exposure of bacteria to a diversity of b-lactams has forced continuous production and mutation of b-lactamases, expanding their activity against the newly developed b-lactam antibiotics (Shah *et al.* 2004). These enzymes, named extended-spectrum b-lactamases (ESBLs), with more than 100 variants of different types, are most frequently reported as SHV, TEM, OXY and CTX-M types (Gniadkowski 2001).

Recent studies have alerted scientists to the worldwide presence of ESBLs in bacteria recovered from a diversity of animals and food products

(Smet *et al.* 2008), with reports of the transfer of antibiotic resistance bacteria and their genes from animals to humans through the food chain (Angulo *et al.* 2004; Phillips *et al.* 2004). In fact, faecal contamination might occur during animal slaughter and/or processing, and the growth of the contaminating bacteria may occur during the product distribution and storage phases (IFT 2006; Simeoni *et al.* 2008). Moreover, lack of hygiene in food manipulation at retail outlets is also responsible for the spread of food pathogenic and commensal bacteria (Gevers *et al.* 2003; Macovei and Zurek 2007; Simeoni *et al.* 2008). In the current EU regulation on microbiological criteria for foodstuffs (Anon 2007) the test for Enterobacteriaceae has replaced the tests for coliforms that traditionally have been used as indicators of hygiene and contamination after processing. This is also valid for the evaluation of the microbiological quality of some RTE foods sampled at the point of sale (Gilbert *et al.* 2000). The awareness of antimicrobial resistance in commensals from food-producing animals lead the World Health Organization and the European Commission to consider their surveillance as one of the main priorities to control the spread of antimicrobial resistance from food animal products to humans (WHO 2000).

Many studies have been carried out on the spread of antimicrobial resistances perpetrated by bacteria isolated in clinical environments (Fernandes *et al.* 2009), but a more restricted number of equivalent studies have been conducted on the role of resistant bacteria in food (Simeoni *et al.* 2008). Reports on the occurrence of b-lactamases among commensal Enterobacteriaceae from RTE food from animal sources are very scarce in Portugal (Amador *et al.* 2009). The aim of this study was to examine the presence of b-lactam-resistant bacteria in different types of Portuguese RTE meat products (deli meats) as well as to investigate the antimicrobial resistance patterns and the presence of the most abundant ESBLs (TEM, CTX, SHV) and OXYgenes within these bacteria.

MATERIALS AND METHODS

Characterization of the Deli Meat Products and Sampling

Nineteen samples, corresponding to 11 types of Portuguese RTE meat (deli meats) products collected in 2007 in the central region of Portugal (Coimbra) were analyzed in this study. With the exceptions of one ham sample, and one meat salad sample, the products were not prepackaged. The deli meats were purchased at two supermarkets, two butchers and one market, and corresponded to pork, turkey or duck meat, and were differently processed (smoked and/or salted and boiled) (Table 1).

TABLE 1.
CHARACTERIZATION OF PORTUGUESE DELI MEATS WITH RESPECT TO PRODUCT TYPE, ANIMAL ORIGIN, PROCESSING TYPE,
PURCHASE SITE AND MEAN VALUES OF AMPICILLIN RESISTANT (AMP^R) BACTERIA (cfu/g) ON VRBG PLATES, AFTER 38 h AT 37C

Product type code	Product type	Animal origin	Processing	Purchase site	N. of samples	CFU/g
A	Ham	Pork *	Boiled	Supermarket 1	4	ND
		Turkey		Supermarket 1		2.33E + 05
		Pork		Butcher 1		ND
		Pork		Butcher 2		1.00E + 05
B	Smoked sausage ¹	Pork	Smoked	Butcher 1	3	<10 ³
				Butcher 2		<10 ³
				Market		ND
C	Salted ribs ²	Pork	Boiled	Butcher 1	1	1.10E + 08
D	Cold cuts ³	Pork	Smoked and salted	Supermarket 2	1	1.00E + 04
E	Smoked and salted ham ⁴	Pork	Smoked and salted	Supermarket 2	3	<10 ³
				Market		ND
				Market		ND
F	Thick sausage ⁵	Pork	Smoked and salted	Market	1	**
G	Industrial meat salad ⁶	Pork*	Boiled	Supermarket 2	2	<10 ³
	Artisanal meat salad ⁷	Pork		Supermarket 2		ND
H	Pâté	Duck	Boiled	Supermarket 2	1	ND
I	Smoked and salted loin ⁸	Pork	Smoked and salted	Market	1	**
J	Black smoked sausage ⁹	Pork	Smoked and salted	Supermarket 2	1	**
L	Large Italian type sausage ¹⁰	Turkey	Boiled	Butcher 2	1	6.67E + 03

* Prepackaged.

** Negative in 25 g.

(1) *Chouriço de carne*; (2) *costela salgada*; (3) assorted parings from *paio*, *salsichão* and ham; (4) *presunto*; (5) *paio*; (6) meat salad with mayonese; (7) *salada de orelha*; (8) *lombo de porco preto*; (9) *moiras*; (10) *mortadela*.

(ND) not determined; cfu, colony-forming unit.

An aliquot of 25 g was removed from each sample and blended for 2 min, in a Stomacher® (Barcelona, Spain), with 225 mL of buffered peptone water (BPW) (pH 7.0) supplemented with 50 mg/mL cycloheximidine (Sigma, St. Louis, MO).

Enumeration of Ampicillin Resistant Bacteria

The enumeration of ampicillin resistant (AMP^r) bacteria was performed on randomly selected colonies on VRBG agar (Violet Red Bile Glucose) agar (Oxoid, Hampshire, England) as follows: portions of 0.1 mL of the homogenized or of the appropriately diluted meat product samples in BPW containing 50 mg/mL cycloheximidine, obtained as described above, were immediately surface plated onto three replicate plates of VRBG supplemented with 20 mg/mL of sodium ampicillin (AppliChem, Damstadt, Germany). Plates were incubated aerobically at 37C for 38 h before counting the colonies. VRBG is a selective medium for Enterobacteriaceae when the sample is double-layered and only the purple (>2 mm) colonies are enumerated after 18 h at 37C. Once the incubation was performed aerobically for 38 h, all the AMP^r isolates were counted.

Detection of Ampicillin-Resistant Bacteria

In order to recover all the resistance phenotypes present in the meat products, each homogenized sample was incubated overnight at 37C, before surface plating on VRBG and on SS (*Salmonella* & *Shigella*) agar (Oxoid). Both media were supplemented with 20 mg/mL of sodium ampicillin. Plates were incubated aerobically at 37C for 24 to 48 h. Different morphological types of AMP^r colonies were selected and the cultures were purified and phenotypically confirmed by re-streaking isolated colonies five times onto the respective isolation medium. Strains *Escherichia coli* XL1-Blue and *Escherichia coli* XL1-Blue transformed with plasmid pUC18 (which contains the TEM-1 b-lactamase gene) were used as negative and positive controls, respectively, for AMP^r phenotype identification.

Nitrocefin Test and Antimicrobial Susceptibility Tests

The detection of b-lactamases produced by the AMP^r isolates was performed by adding nitrocefin (Oxoid, Hants, England) to the cell biomass, according to the manufacturer instructions. For the positive nitrocefin strains, the susceptibility pattern determination was performed by the disk diffusion method on Mueller Hinton agar (Oxoid) with antibiotic disks (Oxoid) according to the Clinical Laboratory Standards Institute (CLSI) (Jorgensen and NCCLS 2003; NCCLS 2005). Thirteen different antibiotics were used: amoxicillin/

clavulanic acid combination (Clavamox) (AMC) 30 mg/10 mg, respectively; ceftazidime (CAZ) 30 mg; cefotaxime (CTX) 30 mg; cefpirome (CPO) 30 mg; aztreonam (ATM) 30 mg; cefoxitin (FOX) 30 mg; imipenem (IPM) 10 mg; meropenem (MEM) 10 mg; chloramphenicol (CHL) 30 mg; tetracycline (TET) 30 mg; gentamicin (GEN) 10 mg; trimethoprim/sulfamethoxazol (Bactrim®; SXT) combination (1:19) and ciprofloxacin (CIP) 5 mg.

In order to observe the influence of clavulanic acid on both CAZ and CTX, these antibiotics were positioned in line, with AMC in the middle. The results of the antimicrobial susceptibilities were interpreted according to the CLSI guidelines.

Bacterial Identification

The identification of b-lactamase producing isolates randomly selected from VRBG and SS plates was carried out in two steps. First, classical morphological and physiological tests were performed in agreement with the Bergey's Manual of Determinative Bacteriology (Holt 1994). These tests included Gram staining reaction, morphology, motility and biochemical tests: fermentative/oxidative metabolism of glucose (Hugh and Leifson 1953) (fermentation of glucose to acid or to acid and gas), aerobic/anaerobic growth, catalase and oxidase reactions. Subsequently, the isolates identified as belonging to the Enterobacteriaceae family were submitted to additional identification by using standard biochemical methods of API 20E (bioMérieux SA, Marcy l'Etoile, France), following the manufacturer instructions.

Detection of b-Lactamase Genes

For the detection of four b-lactamase (*bla*) genes (*bla*_{TEM}, *bla*_{SHV} and *bla*_{CTX-M/bla}_{OXY}) involved in b-lactam resistance a multiplex-PCR approach described by Monstein *et al.* (2007) was used. Genomic DNA extraction was performed with the guanidine thiocyanate method according to Pitcher *et al.* (1989) and plasmid DNA extraction was performed with the Kit *JETquick* Miniprep DNA Genomed (Löhne, Germany). The primers used were synthesized by Metabion International AG (Martinsried, Germany).

Strains *E. coli* UB0402407 and *K. pneumoniae* HY0301692 (kindly provided by the Clinical Microbiology Department, Linköping University, Sweden) were used as positive controls for *bla*_{TEM} + *bla*_{CTX-M/bla}_{OXY} genes and *bla*_{SHV} gene, respectively. The expected sizes of these amplicons were 445 bp for the *bla*_{TEM}, 593 bp for the *bla*_{CTX-M/bla}_{OXY} and 747 bp for the *bla*_{SHV}.

Direct Sequencing of the *bla*_{CTX-M/bla}_{OXY} Amplicons

In order to discriminate between *bla*_{CTX-M} and *bla*_{OXY} genes direct sequencing of the expected size amplicons was performed. Amplified DNA

fragments were purified from the agarose gels using the AxyPrep DNA Gel Extraction kit (Axygen Biosciences, Bioron, Germany), according to the manufacturer recommendations and eluted in 30–50 mL sterile distilled water. Concentration of purified DNA was determined and set to about 1 mg/mL.

The nucleotide sequences were obtained using the DNA sequencing kit, BigDye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems, Foster City, CA) with the primers referred above (Monstein *et al.* 2007). Briefly, 50 ng of DNA was mixed with 10 pmol of forward or reverse primer, 8 ml of a terminator ready reaction mix and sterile distilled water to a final reaction volume of 20 mL. Amplification conditions were 25 cycles of 96C for 10 s, 50C for 10 s and 60C for 4 min. Rapid removal of unincorporated fluorescent dye-terminators from cycle sequencing reactions was carried out prior to sequencing on Performa DTR Ultra 96-Well Plate (EdgeBio, PN 4050207). Sequence data was obtained on an ABI 3730xl Genetic Analyzer (Applied Biosystems).

DNA from each isolate was used to produce the overlapping PCR products, being the respective sequences assembled to generate the final isolate sequence consensus. All the nucleotide sequences in this study were obtained from one unique isolate.

RESULTS AND DISCUSSION

Enumeration of Ampicillin Resistant Bacteria and Detection of β -Lactamases

The enumeration of AMP^r bacteria recovered from the deli meat products was performed on nine samples randomly selected, belonging to seven types of meat products (A, B, C, D, E, G and L) (Table 1). The counting data of AMP^r bacteria on VRBG plates varied from negative in 25 g to 1.0×10^8 colony-forming units (cfu)/g (Table 1). In general, the numbers of AMP^r isolates were lower in smoked products (types B and E). The industrial meat salad (type G), besides being prepacked, had in its formulation different preservatives that could account for the low numbers that can be deduced from the test ($<10^3$ cfu/g).

Antibiotic-resistant microorganisms were also reported at levels ranging from 10^2 to 10^7 CFU/g, in the majority of retail foods examined, in the United States, including raw foods, such as meat and shrimp, and RTE products, such as cheeses and salads (Wang *et al.* 2005).

In order to recover all the resistance phenotypes present in deli meats, even stressed ones because of the processing type, a total of 78 AMP^r isolates were recovered, after the enrichment step, from VRBG and from SS plates.

These isolates were screened for the presence of b-lactamases by the nitrocefin test. All the AMP^r isolates were found to be positive for the presence of b-lactamases. Both media used to recover b-lactam-resistant bacteria appeared to be suitable for this objective, as only one (1.3% of the isolates) and seven (9% of the isolates) from a total of 78 b-lactamase producers subjected to identification were recovered exclusively either from SS or from VRBG plates, respectively (Table 2). No isolates were recovered, after enrichment, from product type F, I and J (thick sausage, smoked and salted loin and black smoked sausage, respectively) (Tables 1 and 2).

Bacterial Identification

Of the 78 nitrocefin positive isolates (46 from VRBG and 32 from SS plates), 87.2% were identified as belonging to the Enterobacteriaceae family, 9.0% to the *Pseudomonas* genus and 3.8% to the *Aeromonas* genus (Table 2). Within the Enterobacteriaceae family, 42 isolates (16 from SS and 26 from VRBG plates) were identified as belonging to 11 different species. It was not possible to identify 26 of the Enterobacteriaceae isolates (33.3%) recovered from both media to the species level. The type of meat product D (cold cuts) was the source of the highest number of identified Enterobacteriaceae (26 isolates corresponding to five different species). This might be ascribed to the fact that it was composed of parings of three types of deli meat. After the enrichment step, six different species were collected from the artisanal meat salad (product type G), namely, *Morganella morganii*, *Enterobacter cloacae*, *K. oxytoca*, *K. pneumoniae* spp. *pneumoniae*, *Pseudomonas aeruginosa* and *P. fluorescens* (Table 2).

The criteria listed for Enterobacteriaceae in the PHLS document (Gilbert *et al.* 2000) regarding the microbiological quality of RTE foods sampled at the point of sale do not apply to fresh fruit and vegetables or to sandwiches containing salad vegetables because fresh fruit and vegetables often carry high levels of these organisms as part of their normal flora. For the other categories of RTE foods (including the type of deli meats analyzed here) food products presenting Enterobacteriaceae counts that exceed 10⁴ cfu/g are considered unsatisfactory (Gilbert *et al.* 2000). As 87.2% of the identified isolates resided within the Enterobacteriaceae family, the samples submitted for enumeration corresponding to product types A, C and D, according to these guidelines would probably have been considered unsatisfactory (Table 1). As Enterobacteriaceae are used as a parameter in process hygiene criteria, the results presented here suggest a lack of hygiene at one or more of the processing stages of these products and/or growth during distribution and storage at retail outlets. In order to avoid deli meat contamination with these and other potential hazardous bacteria, the existence of good sanitary conditions during

TABLE 2.
IDENTIFICATION OF AMPICILLIN RESISTANT ISOLATES POSITIVE FOR THE NITROCEFAN TEST, AND RESPECTIVE TYPE OF DELI
MEAT AND RECOVERY MEDIUM

Family/genus	Species	N. of isolates	%	Type of meat product	Recovery medium
Enterobacteriaceae	NI	26	33.3	A, D, E, H, L	VRBG, SS
	<i>Cedecea davisae</i>	2	2.6	H	VRBG
	<i>Escherichia coli</i>	12	15.4	B, C, D	VRBG, SS
	<i>Morganella morganii</i>	2	2.6	D, G	VRBG, SS
	<i>Enterobacter cloacae</i>	4	5.1	A, C, G	VRBG, SS
	<i>Escherichia vulneris</i>	4	5.1	B, C	VRBG, SS
	<i>Klebsiella oxytoca</i>	6	7.7	A, D, E, G	VRBG, SS
	<i>K. pneumoniae</i> ssp. <i>ozaenae</i>	1	1.3	B	VRBG
	<i>Enterobacter amnigenus</i>	3	3.8	A, E	VRBG
	<i>Enterobacter aerogenes</i>	1	1.3	A	VRBG
	<i>K. pneumoniae</i> ssp. <i>pneumoniae</i>	6	7.7	B, D, E, G	VRBG, SS
	<i>Serratia liquefaciens</i>	1	1.3	H	SS
	NI	2	2.6	B, G	VRBG, SS
<i>Pseudomonas</i>	<i>Pseudomonas aeruginosa</i>	2	2.6	G	VRBG, SS
	<i>Pseudomonas fluorescens</i>	3	3.8	B, G	VRBG, SS
<i>Aeromonas</i>	NI	3	3.8	A	VRBG, SS
All		78	100	A, B, C, D, E, G, H, L	VRBG, SS

VRBG, Violet Red Bile Glucose agar; SS, *Salmonella/Shigella* agar; NI, not identified; A, ham; B, smoked sausage; C, salted ribs; D, cold cuts; E, smoked and salted ham; F, thick sausage; G, meat salad; H, pâté; I, smoked and salted loin; J, black smoked sausage; L, large Italian type sausage.

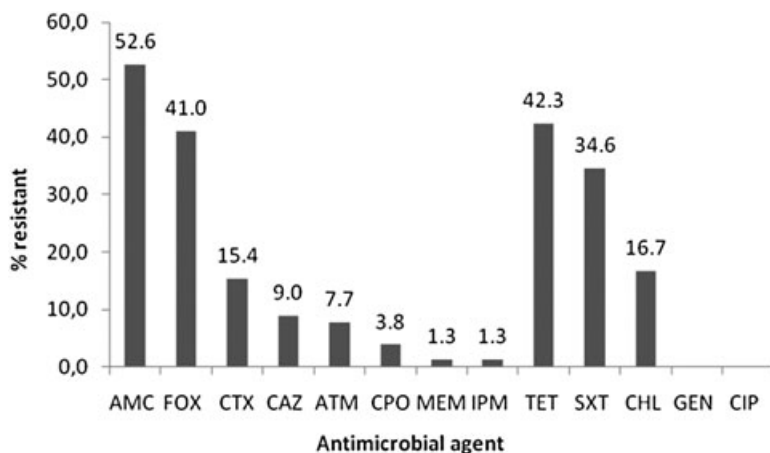


FIG. 1. PERCENTAGE OF THE RESISTANT PHENOTYPE FOR THE ANTIMICROBIAL AGENTS TESTED AMONG THE ISOLATED AMPICILLIN RESISTANT/NITROCEFEN POSITIVE DELI MEAT ISOLATES

The antimicrobial agents tested were the b-lactams: AMC, amoxicillin/clavulanic acid (30:10 mg); FOX, cefoxitin (30 mg); CTX, cefotaxime (30 mg); CAZ, ceftazidime (30 mg); ATM, aztreonam (30 mg); CPO, cefpirome (30 mg); MEM, meropenem (10 mg); IPM, imipenem (10 mg); and the non-b-lactams: TET, tetracycline (30 mg); SXT, trimethoprim/sulfamethoxazole (1:19 mg); CHL, choranfenicol (30 mg); GEN, gentamicin (10 mg); and CIP, ciprofloxacin (5 mg).

animal slaughtering and meat processing are obligatory. This includes herd health management and hygiene along the entire production chain.

Antimicrobial Susceptibility Tests

The determination of antimicrobial susceptibility and resistance for the 13 antimicrobial agents revealed a higher resistance in the b-lactams group AMC (52.6%) and FOX (41.0%) and in the non-b-lactam group TET (42.3%), followed by SXT (34.6%) (Fig. 1). Conversely, the most effective antimicrobials were GEN and CIP (100% each) and IPM and MEM (98.7% each) (Fig. 1).

The antimicrobial susceptibility of the meat isolates to these 13 antimicrobial agents was compared with bacterial isolates from cheese in a previous study (Amador *et al.* 2009). The antimicrobial susceptibility data obtained in the present study were similar to those described for isolates recovered from cheese samples that showed values of antimicrobial resistance to AMC, FOX, TET and SXT of 51.7, 46.5, 46.5 and 38.4%, respectively. Similarly, the most efficient antimicrobials were GEN (98.3%), CIP (97.7%), IPM (95.3%) and MEM (90.7%).

A possible explanation for the high incidence of resistance observed in meat products predominantly made of pork might be the intensive use in pig farming of antibiotics including streptomycin, penicillin, amoxicillin/clavulanic acid, oxytetracycline, tylosin, colistin, trimethoprim/sulfamethoxazol, tiamotin, among others (Silbergeld *et al.* 2008). In fact, meat isolates are possibly a result of enterobacterial contamination during slaughtering and have therefore been submitted to selective pressure of the antimicrobial agents used.

The high frequency of resistance to b-lactams, such as penicillins and their inhibitors, cephalosporins and cefoxitin may be explained by the extensive use in Portugal. Latest data from *The National (Portuguese) Authority of Medicines and Health Products* (INFARMED, I.P.), kindly provided by this organization, indicate that b-lactams were still the most sold group in this country, reaching a total of 49.2% (data not shown) of all antibiotic units (packages) according to the report of 2008.

The high resistance detected to TET (42.3%), similar to levels previously obtained in cheese isolates (46.5%) (Amador *et al.* 2009), may be related to the extensive use of TET in veterinary practice (Emborg *et al.* 2007; Stine *et al.* 2007; Nawaz *et al.* 2008). Nevertheless, it could also mean an eventual non-efficiency in humans through selection, following the widespread use of these antibiotics (Nawaz *et al.* 2008). These results are also in agreement with the work of Simeoni *et al.* (2008), where from an entire swine production chain, 72% of the *Staphylococcus* isolates had two types of resistance determinants to tetracycline, namely tet (K) and tet (M) genes, showing a wide spread of this type of resistance.

Curiously, the high bacterial susceptibility to CIP, already reported by Amador *et al.* (2009) in Portugal, is in contrast with reports from other European countries, where this type of antibiotics is one of the most frequently used (Ferech *et al.* 2006).

A multidrug-resistant (MDR) phenotype was defined for isolates resistant to three or more antimicrobial agents that were structurally unrelated. This analysis revealed that 35.9% of the different phenotypes detected displayed a MDR phenotype (Table 3). The most prevalent resistance phenotypes were AMP (21.8%), AMP + SXT + TET (11.5%), AMP + AMC (10.3%) followed by the AMP + TET and AMP + AMC + FOX (each 7.7%).

In a previous study by Amador *et al.* (2009) regarding resistant isolates from cheese, the relative frequency of a particular resistance phenotype was similar to the values found in this study with isolates with the same resistance profile.

Relative frequency of MDR phenotypes detected among deli meat isolates found in the present study was quite elevated (35.9%) and similar to the value observed among cheese isolates (31.4%) (Amador *et al.* 2009). These

TABLE 3.
RELATIVE FREQUENCY OF RESISTANCE PHENOTYPES AND GENOTYPES DETECTED
AMONG DELI MEAT ISOLATES

	Resistance phenotype	Phenotype		Genotype			
		N° isolates	RF	TEM		CTX-M/OXY	
				(a)	(b)	(a)	(b)
Multidrug resistant (MDR) phenotypes	AMP, SXT, TET	9	11.5	100.0 (9/9)	39.1	0.0 (0/9)	0.0
	AMP, AMC, FOX, CHL, TET	4	5.1	50.0 (2/4)	8.7	0.0 (0/4)	0.0
	AMP, AMC, FOX, CHL, SXT	2	2.6	0.0 (0/2)	0.0	0.0 (0/2)	0.0
	AMP, AMC, FOX, CEPH3, CHL, SXT, TET	2	2.6	0.0 (0/2)	0.0	0.0 (0/2)	0.0
	AMP, AMC, FOX, SXT, TET	2	2.6	50.0 (1/2)	4.3	0.0 (0/2)	0.0
	AMP, CHL, SXT, TET	2	2.6	100.0 (2/2)	8.7	0.0 (0/2)	0.0
	AMP, AMC, CEPH3, CPO, CHL, SXT, TET	1	1.3	0.0 (0/1)	0.0	0.0 (0/1)	0.0
	AMP, AMC, CEPH3, CPO, CHL, TET	1	1.3	100.0 (1/1)	4.3	0.0 (0/1)	0.0
	AMP, AMC, FOX, CEPH3, CPO, ATM, SXT	1	1.3	0.0 (0/1)	0.0	0.0 (0/1)	0.0
	AMP, AMC, FOX, CEPH3, SXT, TET	1	1.3	0.0 (0/0)	0.0	0.0 (0/0)	0.0
	AMP, AMC, SXT, TET	1	1.3	100.0 (1/1)	4.3	0.0 (0/1)	0.0
	AMP, FOX, CHL, SXT	1	1.3	0.0 (0/1)	0.0	0.0 (0/1)	0.0
	AMP, FOX, SXT, TET	1	1.3	0.0 (0/1)	0.0	0.0 (0/1)	0.0
	Sub-total	28	35.9	59.3 (16/27)	69.6	0.0 (0/27)	0.0
Non-MDR phenotypes	AMP	17	21.8	15.4 (2/13)	8.7	30.8 (4/13)	100.0
	AMP, AMC	8	10.3	0.0 (0/7)	0.0	0.0 (0/7)	0.0
	AMP, TET	6	7.7	0.8 (4/5)	17.4	0.0 (0/5)	0.0
	AMP, AMC, FOX	6	7.7	0.0 (0/5)	0.0	0.0 (0/5)	0.0
	AMP, AMC, FOX, CEPH3, ATM	5	6.4	20.0 (1/5)	4.3	0.0 (0/5)	0.0
	AMP, AMC, FOX, SXT	2	2.6	0.0 (0/1)	0.0	0.0 (0/1)	0.0
	AMP, FOX, TET	2	2.6	0.0 (0/1)	0.0	0.0 (0/1)	0.0
	AMP, AMC, FOX, CEPH3	1	1.3	0.0 (0/1)	0.0	0.0 (0/1)	0.0
	AMP, AMC, FOX, CEPH3, SXT	1	1.3	0.0 (0/1)	0.0	0.0 (0/1)	0.0
	AMP, AMC, FOX, TET	1	1.3	0.0 (0/0)	0.0	0.0 (0/0)	0.0
	AMP, AMC, SXT	1	1.3	0.0 (0/1)	0.0	0.0 (0/1)	0.0
	Subtotal	50	64.1	17.5 (7/40)	30.4	10.0 (4/40)	100.0
	Total	78	100	34.3 (23/67*)	100.0	6.0 (4/67*)	100.0

* Eleven out of 78 isolates irreversibly lost viability. For this only 67 isolates were tested for the presence of the b-lactamases genes.

(a) Relative frequency of *bla*TEM or *bla*CTX-M/OXY positive results in mPCR/number of tested isolates; in brackets, number of *bla*TEM or *bla*CTX-M/OXY positive results in mPCR/number of tested isolates. (b) Relative frequency of *bla*TEM or *bla*CTX-M/OXY positive results in mPCR/ total number of positive results in mPCR tests.

Antimicrobials: AMC, amoxicillin/clavulanic acid; AMP, ampicillin; ATM, aztreonam; CHL, chorafenicol; CPO, cefpirome; FOX, cefoxitin; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; CEPH3, third cephalosporins (cefotaxime and/or ceftazidime); RF, relative frequency of meat isolate's resistance phenotype.

elevated MDR phenotypes are also in agreement with the results of Fernandes *et al.* (2008) that reported the spreading of MDR in Portuguese hospitals and in the community by isolates encoding for ESBLs. Increased prevalence of MDR phenotypes were also found all over Europe (Livermore *et al.* 2007).

Detection of b-Lactamase Genes

Seventy eight randomly selected ampicillin resistant isolates were investigated for resistance phenotype pattern and genus/species identification. Nevertheless, 11 isolates irreversibly lost viability. The remaining 67 isolates were then tested for the presence of four b-lactamases genes with multiplex-PCR (Table 3). This analysis showed that 34.3% (23/67) of the isolates harbored a gene coding for the TEM-type enzyme (*bla*_{TEM}) and 6.0% (4/67) of the isolates harbor a gene for the CTX-M/OXY-type enzymes (*bla*_{CTX-M} and *bla*_{OXY}, respectively). No *bla*_{CTX-M} and *bla*_{SHV} were detected among the isolates under study (Table 3).

The *bla*_{TEM} gene was more frequently detected (59.3%, 16/27) among isolates with MDR phenotypes, than among those with non-MDR phenotypes (17.5%, 7/40), although a higher number of isolates of the latter group were tested. The *bla*_{TEM} gene was detected in all the isolates with AMP + SXT + TET resistance profile.

The high number of isolates with *bla*_{TEM} genes might be explained by the wide dissemination of plasmids coding for TEM enzymes, sometimes in mobile genetic elements, which are responsible for the broad-spectrum resistance phenotype to penicillin in many gram-negative bacteria, namely *E. coli*, in clinical isolates (Schmitt *et al.* 2007). Recently, an alarming level of TEM frequency was reported in Chinese chicken farms (Yuan *et al.* 2009).

The presence of *bla*_{TEM} in bacteria isolated from deli meats reported in this study, is in agreement with several recent reports showing that TEM b-lactamases are particularly involved in resistance phenotypes presented by bacteria isolated from several agro-industrial environments such as livestock (Kim *et al.* 2005; Cloeckaert *et al.* 2007; Sunde *et al.* 2009). The presence of TEM b-lactamases in these bacteria may be explained by its insertion between transposable elements that move rapidly through bacterial populations (Partridge and Hall 2005) and may become a major public health concern. Several studies have reported this transfer of antimicrobial resistance genes from food bacteria to human flora in the food chain (Angulo *et al.* 2004; Phillips *et al.* 2004; Simeoni *et al.* 2008).

The pair of primers used to amplify *bla*_{CTX} was proved to also target the *bla*_{OXY} gene, therefore detecting both without discrimination, for which sequencing is required (Monstein *et al.* 2007; Monstein *et al.* 2009). A BLAST search for similarity with the sequences produced revealed no similarity between *bla*_{CTX-M} and *bla*_{OXY} (data not shown).

In the present study, *bla*_{OXY} genes were exclusively detected in the AMP resistance profiles of four isolates of *K. oxytoca* designated as 3C (from cold cuts), 35C (from meat salad), 36C (from smoked and salted ham) and 76C (from meat salad). This organism is usually reported as a clinical isolate.

These results are not consistent with the phenotypes of resistance that have been found in isolates of *K. oxytoca* resistant to broad-spectrum cephalosporins and to monobactam (except ceftazidime, cephamycins and carbapenems), which represent overproduction of β -lactamases chromosomally encoded, because of some mutations in the promoter regions -35 and -10 (Mammeri *et al.* 2003; Schmitt *et al.* 2007).

The sequences of the amplicons from the four *K. oxytoca* isolates were deposited in the EMBL/GenBank/ DDBJ database with the accession numbers: GQ433982 (isolate 3C), GQ433983 (isolate 35C), GQ433980 (isolate 36C) and GQ433981 (isolate 76C).

These sequences were then compared with others, from clinical reference strains of *K. oxytoca* recovered at hospitals in Canada, Denmark, France, Sweden, Switzerland and the Netherlands (Fournier *et al.* 1995; Wu *et al.* 1999; Granier *et al.* 2003; Fevre *et al.* 2005). The sequences from these clinical isolates are representative of the six groups of the OXY β -lactamase (Fevre *et al.* 2005) and were retrieved from EMBL/GenBank/DDBJ database. The nucleotide sequences GQ433980 and GQ433981 were more similar (within 98% and 99%) to sequences (Y17714, Z49084, FM178458 and FM178457), whereas the GQ433982 was more similar (97%) to sequences (AJ871871 and AJ871872) and GQ433983 was more similar (98%) to sequences (AY077482 and Z30177). Therefore, based on the available data, three *bla*_{OXY} genes were identified namely the *bla*_{OXY-2} gene in isolates 36C and 76C, the *bla*_{OXY-5} gene in isolate 3C and the *bla*_{OXY-1} gene in isolate 35C.

The alignment of the deduced *bla*_{OXY} aminoacidic sequences with the others referred to above (Fig. 2) showed that aminoacidic sequences deduced from the sequences of isolates 36C and 76C were similar to sequences Y17714 (98 and 99%), Z49084 (98 and 99%), FM178458 (98 and 98%) and FM178457 (99 and 98%), respectively.

The deduced sequence from isolate 3C is 98% similar to OXY-5 sequences AJ871871 and AJ871872, of strains SB2933 and SB2942 and the 35C is similar to OXY-1 sequences AY077482 (99%) and Z30177 (100%), obtained from strain SG12 and reference strain SL781.

The isolates carrying chromosomal *bla*_{OXY}, as described in the present study, may only represent a risk to public health if a genetic transfer mechanism from chromosome to plasmid takes place, as occurred with other β -lactamase genes. Several studies have shown that resistance chromosome genes may be transferred to plasmids by recombination mechanisms. For example, Bonnet (2004) suggested the mobilization of *bla*_{CTX-M} genes from the

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      EV          TV          WV          SW          EW          *
3-AP491278: .T...S..I..-L...C.S...N=-...ST...D.T.N..I...KD...AS...V... : 135
2-Y17714 : .I.ML...A...T..H..T...N..I...S...K...NA...T... : 136
2-Z49084 : .I.ML...A...T..H..T...N..I...S...K...NA...T... : 137
FM178458 : .I.ML...SA...T..H..T...N..I...S...K...NA...T... : 137
FM178457 : .I.ML...SA...T..HX..T...R...N..I...S...K...NA...T... : 137
76C : .....K...NA...T... : 51
36C : .....K...NA...T... : 68
FM178422 : .L...RS...H.D...S... : 137
6-AJ871879: .L...S...N...H.D...S... : 138
5-AJ871871: .L...H...X...G... : 137
5-AJ871872: .L...H...X...G... : 137
3C : .....X...S... : 53
4-AY077481: .L...S...TL...H.D...S... : 137
1-AY077482: .L...S... : 138
1-Z30177 : .L...S... : 138
35C : .....X...S... : 69
MiKsSWRktAlmAAaavPLLLaSgsLWAsadaiqqKladLEKrsGRLGVALInTaDdSQtLYRGDERFAMCSTgKVMAAAAVLKQSESnpeVvNKRLEIkkDLVVMSp1TEKHLQSGMTLAELSaaLQYSDNTAM

      40          *          160          *          180          *          200          *          220          *          240          *          260          *
3-AP491278: ..M.....R.....T.....E.....H.....V.....L..... : 272
2-Y17714 : .L.....R.....A.....T.....E.....S.....D.....E..V.....D.....N : 273
2-Z49084 : .L.....R.....A.....T.....E.....S.....D.....E..V.....D.....N : 274
FM178458 : .L.....R.....A.....T.....E.....S.....D.....E..V.....D.....N : 274
FM178457 : .L.....R.....A.....T.....E.....S.....D.....E..V.....D.....EG.N : 274
76C : .L.....R.....A.....T.....E.....S.....D.....E..V.....D.....I..... : 182
36C : .L.....R.....A.....T.....E.....S.....E..V.....I..... : 199
FM178422 : ..M.S..... : 274
6-AJ871879: ..M.S..... : 275
5-AJ871871: .....L.....M..... : 274
5-AJ871872: .....M..... : 274
3C : .....M.....G..... : 184
4-AY077481: .....M.....G..... : 274
1-AY077482: ..M.S.....G..... : 275
1-Z30177 : ..M.S.....G..... : 275
35C : ..M.S..... : 199
NkiIgYLGgPEKVTAFaQsIGDvTFRLDrtEPaLNsaIPGdkRDTtPLAMAEsLrKLTLGnALGEQQRaQLVTLKGNTTGGQSIRAGLPaSWaVGDKtGaGDYGT TN0IAVWPEnHAPLvLVTYFTQpQdaKs

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FIG. 2. DEDUCED AMINOACIDIC SEQUENCE ALIGNMENT OF THE *bla*_{oxy} GENE/CODING REGION, OBTAINED FROM FOUR ISOLATES (76C, 36C, 35C, 3C) AND 12 SEQUENCES RETRIEVED FROM EMBL/GENBANK/DBJ DATABASE (Y17714, Z49084, FM178458, FM178457, FM178422, AJ871879, AJ871872, AJ871871, AY077481, AY077482, Z30177 AND AF491278)

Hyphens represent gaps included in the sequence.

chromosomes of environmental *Kluyvera* bacteria to plasmids. That is why conjugation and transformation studies are still performed in order to understand possible transmission mechanisms of these genes (Fournier *et al.* 1996; Granier *et al.* 2003; Mammeri *et al.* 2003; Decré *et al.* 2004).

To our knowledge, this is the first report in Portugal of the presence of b-lactam resistant bacteria in deli meats, a type of RTE food product. This is also the first Portuguese report describing the presence of *K. pneumoniae* ssp. in food. The data presented here confirm previous statements that food can contribute to the spread of antibiotic resistant Enterobacteriaceae in the community (Prats *et al.* 2003) namely by transfer of b-lactam-resistant bacteria to the gastro-intestinal (GI) flora of the consumer. This would also accentuate the importance of good hygienic practices during food animal slaughtering and/or processing, distribution and storage.

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