In vitro transference and molecular characterization of *bla*$_{\text{TEM}}$ genes in bacteria isolated from Portuguese ready-to-eat foods

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Abstract The principal aim of this study was to investigate the possibility of transference to *Escherichia coli* of β-lactam resistance genes found in bacteria isolated from ready-to-eat (RTE) Portuguese traditional food. From previous screenings, 128 β-lactam resistant isolates (from different types of cheese and of delicatessen meats), largely from the Enterobacteriaceae family were selected and 31.3% of them proved to transfer resistance determinants in transconjugation assays. Multiplex PCR in donor and transconjugant isolates did not detect *bla*$_{\text{CTX}}$, *bla*$_{\text{SHV}}$ and *bla*$_{\text{OXY}}$, but *bla*$_{\text{TEM}}$ was present in 85% of them, while two new TEMs (TEM-179 and TEM-180) were identified in two isolates. The sequencing of these amplicons showed identity between donor and transconjugant genes indicating in vitro plasmid DNA transfer. These results suggest that if there is an exchange of genes in natural conditions, the consumption of RTE foods, particularly with high levels of Enterobacteriaceae, can contribute to the spread of antibiotic resistance.

Keywords Ready-to-eat (RTE) food · β-lactam-resistant Enterobacteriaceae · *bla*$_{\text{TEM-1}}$ gene · Transconjugants

Introduction

Antimicrobial resistance is an inevitable consequence of the evolutionary adaptation of microbes, but the human use and misuse of antimicrobial drugs have driven the increasingly rapid and prevalent emergence of resistance in a range of pathogenic and commensal microorganisms (Silbergeld et al. 2008). Acquired resistance to an antimicrobial drug can be due either to mutation of a resistance gene or to horizontal gene transfer, through the incorporation of such genes, located in mobile genetic elements such as plasmids, transposons or integrons/gene cassettes, by acceptor bacteria (Catry et al. 2003). The usual ways bacteria use to transfer their genes are via conjugation, transformation or transduction (Schwartz and Chaslus-Dancla 2001), the former being the most important transfer mechanism (Catry et al. 2003).

More than 300 natural extended-spectrum β-lactamase (ESBLs) variants have been identified since the mid-1980s, but the most abundant are represented by SHV, TEM and CTX-M (Gniadkowski 2008; Lahey Clinic 2010).

TEM- and SHV-type ESBLs have developed from their ancestors TEM-1/-2 and SHV-1, respectively, by point mutations, whereas CTX-M appears to have originated from chromosomal β-lactamase genes from *Kluyvera* spp. (Novais et al. 2010). The plasmid-encoded TEM-1/-2 enzymes are widespread and are the cause of resistance to broad-spectrum penicillin in many gram-negative rods (Livermore 1995). SHV-1, the chromosomally encoded β-lactamase K2 from *Klebsiella pneumoniae*, has also been found to be plasmidic in other species (Chang et al. 2001). For both TEM-1 and SHV-1, association with transposons is recognized (Schmitt et al. 2007). Plasmids are responsible for the spread of most of the new β-lactamases, which may also have chromosomal origins (Jacoby and Munoz-Price 2005; Gupta 2007).

Limited information exists about the transfer of resistant pathogenic bacteria and their genes between animals and humans, but even less is known about the potential for exchange with or among commensal bacteria (IFT 2006). However, a high frequency of resistance gene transfer has been demonstrated in in vitro experiments between: gram-negative bacteria, gram-positive bacteria (Courvalin 1994) and the bacterial microbiota of animals and humans (Catry et al. 2003). In a similar way, in vivo experiments between swine and human *Enterococcus faecium* (in the digestive tract of gnotobiotic mice), suggested that gene exchange under natural conditions might also be very frequent (Moubareck et al. 2003). These transmission mechanisms enable fast and efficient gene spread, possibly representing an important aspect of the ecology of antibiotic resistance determinants, which lead to serious public health and environmental problems (Macovei and Zurek 2007).

Nowadays, it is accepted that the commensal Enterobacteriaceae present in the grastrointestinal tract (GI) of humans and of food-producing animals, might represent an important reservoir of antibiotic resistance genes. These GI are favourable sites for horizontal intra- and inter-species gene transfer (Shoemaker et al. 2001), among permanent and transient colonizers, ingested with food and drinks (Smet et al. 2008).

Multiple studies have focused on the characterization of β-lactamases in human clinical *Escherichia coli* isolates, but very few have been performed with isolates of other origins such as healthy humans, animals (healthy or sick) or foods (Briñas et al. 2002). Nevertheless, the food chain, mainly animal food consumption, as well as direct contact with livestock has been identified as a route to spread resistance in the community, either through transfer of resistant bacteria to humans or of their resistance genes to pathogens (Angulo et al. 2004; Phillips et al. 2004). Some studies have evidenced meat processing as the most important aspect in the spreading of resistant bacteria, pathogenic or commensal (Simeoni et al. 2008). Ready-to-eat (RTE) foods, namely cheese and delicatessen meat, although usually containing low levels of *E. coli,* might represent a potential resistance genes source, as they are consumed uncooked (Johnson et al. 2005).

The aim of this study was to search for β-lactam resistance genes (bla_{TEM}, $bla_{\text{CTX-M}}$, bla_{SHV} and bla_{OXY}) in bacteria isolated from Portuguese traditional cheeses and delicatessen meats. The resistance gene transfer ability was investigated in in vitro transconjugation assays with *Escherichia coli*.

Materials and methods

Transconjugation assays

The transconjugation assays were carried out with 128 ampicillin resistant (Amp^{R}) and nitrocefin positive isolates collected from Portuguese RTE traditional food (57 from cheese samples and 71 from delicatessen meats) over 2 years (2007–2008) as previously described (Amador et al. 2009, 2010). *E. coli* J53 (Az^{R}) and *E. coli* HB101 (Stp^{R}) (Bio-Rad, München, Germany) were used as recipient strains in the transconjugation experiments. The liquid mating method used in this work was adapted from Van et al. (2007) as follows: donor and recipient strains freshly grown separately overnight without shaking on trypticase soy broth (TSB) were mixed in a donor-to-recipient ratio of 1:10 in TSB. Mixtures were incubated at 37°C for 18 h without shaking. Then, transconjugants were selected by spreading 100 µl of each mixture onto trypticase soy agar (TSA) containing, according to the recipient strain used, 200 µg of sodium azide/ml and 20 µg of ampicillin/ml, or 100 µg of streptomycin sulfate/ml and 20 µg of ampicillin/ml as selective agents. Plates were aerobically incubated overnight at 37°C for 24 h and the colonies were further re-streaked onto similar media to confirm the phenotype.

For each single test, controls were simultaneously performed for culture media, antibiotic efficiency and donor strains, using different antibiotic supplementation (only 20 µg of ampicillin/ml or only 100 µg of streptomycin sulfate/ml or only 200 µg of sodium azide/ml).

Antimicrobial susceptibility testing

The susceptibility pattern of donor and transconjugants isolates was determined by the disk diffusion Kirby Bauer method described by Amador et al. (2009). Briefly,

Mueller–Hinton agar (Oxoid, Hampshire, England) with antibiotic disks (Oxoid, Hampshire, England) was used according to the Clinical Laboratory Standards Institute (CLSI) recommendations (CLSI 2007). Donor isolates were retested simultaneously with transconjugants, in order to confirm the results.

The isolates were tested to amoxicillin/clavulanic acid combination (AMC) 30 μg/10 μg, respectively; ceftazidime (CAZ) 30 μg; cefotaxime (CTX) 30 μg; cefpirome (CPO) 30 μg; aztreonam (ATM) 30 μg; cefoxitin (FOX) 30 μg; imipenem (IPM) 10 μg; meropenem (MEM) 10 μg; chloramphenicol (CHL) 30 μg; tetracycline (TET) 30 μg; gentamicin (GEN) 10 μg; trimethoprim/sulfamethoxazol (SXT) combination (1:19) and ciprofloxacin (CIP) 5 μg. Strains of *E. coli* ATCC25922 (Liofilchem S.R.L., Roseto degli Abruzzi, Italy), *E. coli* J53 (Az^R) and *E. coli* HB101 (Stp^R) were used as quality controls in minimal inhibitory concentration (MICs) assays.

DNA extraction and PCR amplification

Genomic DNA was extracted according to Amador et al. 2009 and the plasmid DNA extraction was performed with the Kit *JETquick*, Miniprep DNA Genomed (GmbH, Löhne, Germany). Both extractions were carried out at least in duplicate for donor and transconjugant bacteria. DNA fragments were visualized under u.v. light in 1% (m/v) agarose gel using two molecular markers, 100 bp plus 1.5 Kb (BioRon) and λHind III (Sigma).

The detection of genes involved in β-lactam resistance was performed, using genomic and plasmidic DNA of donor and transconjugant strains, by a multiplex-PCR approach for β-lactamase genes (bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}/bla_{OXY}$) according to the procedure described by Monstein et al. (2007). The primers used, synthesised by Frilabo, Metabion International AG, are described in Table 1. Strains used as positive controls in PCR amplifications were: *E. coli* HY0401091 (for CTX-M-2), *E. coli* UB0402407 (for CTX-M-9 and TEM), *K. pneumoniae* HY0301692 (for CTX-M-1, TEM and SHV), *K. pneumoniae* AA0404346 and *K. pneumoniae* MR0500681 (for SHV). Negative controls without DNA were always included.

Direct sequencing of PCR products

The pair of primers TEM-F (5′-ATAAAATTCTTGAAGACGAAA-3′) and TEM-R (5′-GACAGTTACCAATGCTTAATC-3′) (Weill et al. 2004) was used to amplify a 1,080 bp fragment of bla_{TEM} genes. The amplicons with the expected size were purified from agarose gel using the AxyPrep DNA Gel Extraction kit (Axygen Biosciences, Bioron-Germany), according to the manufacturer's recommendations and eluted in 30–50 μl sterile distilled water. Concentration of purified DNA was determined and diluted to achieve about 1 μg of DNA μl^{-1}.

The nucleotide sequences were obtained by direct sequencing of purified PCR products using the DNA sequencing kit, BigDye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems) with the primer set TEM-F/TEM-R. Briefly, the DNA (50 ng) was mixed with 1 μl (10 pmol) of forward or reverse primer, 8 μl of a terminator ready reaction mix and sterile distilled water to a final reaction volume of 20 μl. Amplification conditions were 25 cycles of 96°C for 10 s, 50°C for 10 s and 60°C 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).

Sequencing analysis

From each donor and transconjugant isolates, both DNA strands of the amplicons were used to assess sequence variability. DNA from each isolate was used to produce the overlapping PCR products, being the respective sequences assembled to generate the final isolate sequence consensus. Each nucleotide sequence in this study was obtained from one unique isolate.

For the analysis made in this study 100 TEM non-redundant and complete sequences were retrieved from the EMBL/GenBank/DDBJ and according to the information in Lahey Clinic (2010) and The LacTamase Engineering Database (LACED 2010).

Table 1 Primers used for PCR detection of *bla* genes

Primer	Sequence (5′ → 3′)	Amplicon size (bp)	Gene family
SHV-F	ATGCGTTATATTCGCCTGTG	747	SHV
SHV-R	TGCTTTGTTATTCGGGCCAA		
TEM-F1	TCGCCGCATACACTATTCTCAGAATGA	445	TEM
TEM-R2	ACGCTCACCGGCTCCAGATTTAT		
CTX-M-F	ATGTGCAGYACCAGTAARGTKATGGC	593	CTX-M/OXY
CTX-M-R	TGGGTRAARTARGTSACCAGAAYCAGCGG		

F—forward, R—reverse

CLUSTAL X v.1.81 (Thompson et al. 1997), with the default parameters settings, was used to align consensus sequences.

Results and discussion

Detection and transfer of resistance genes

The transconjugation assays carried out with the 128 isolates (57 from cheese and 71 from delicatessen meat) previously characterized (Amador et al. 2009; Amador et al. 2010) yielded 40 transconjugants (20 from cheese and 20 from delicatessen meat). The respective donor isolates are described in Table 2. Most of the isolates that proved transconjugation (92.5%) belonged to the Enterobacteriaceae family. Among these, three species (*Escherichia coli, Enterobacter cloacae* and *Morganella morganii)* were identified both from cheese and from delicatessen meat products. The most prevalent transconjugant species isolated from both RTE products was *E. coli* (42.5%). These results are consistent with several studies that indicate frequent contamination with antimicrobial-resistant *E. coli,* originating from various RTE foods in the United States (Johnson et al. 2005).

The multiplex-PCR approach for the detection of β-lactamases (bla_{TEM}, bla_{SHV} and $bla_{\text{CTX-M}}$/bla_{OXY}) performed in donor and a respective transconjugant cells was not able to detect the bla_{SHV} (Fig. 1a). Similarly, this gene was also not detected in the isolates that did not transconjugate (data not shown). The same approach confirmed the presence of the bla_{TEM} gene in 85% (34/40) of the cheese and delicatessen meat isolates (Tables 3, 4). Others studies have also showed the prevalence of bla_{TEM} genes in ampicillin-resistant *E. coli* isolates recovered from animal foods and feces of healthy humans and animals (Briñas et al. 2002).

Eighty-five percent (17/20) of cheese isolates proved to transfer the bla_{TEM} gene through transconjugation. As expected, the bla_{TEM} was not detected in the unique *Klebsiella oxytoca* isolated from cheese (isolate 138Q), as *K. oxytoca* usually carries the bla_{OXY} (Fevre et al. 2005). Eighty-five percent (17/20) of the meat isolates transferred the bla_{TEM} gene through transconjugation. Moreover, in the majority (64%) of the delicatessen meat isolates that did not transconjugate the bla_{TEM} gene was detected (data not shown).

The universal degenerated CTX-M primers used also amplify the chromosomal bla_{OXY} (K1-genes in *Klebsiella* spp. and K1-like genes in other Enterobacteriaceae) (Monstein et al. 2009). Although no amplicons were obtained with this primer set in isolates that proved transconjugation, five amplicons were obtained among non-transconjugant isolates. Their sequencing proved the absence of $bla_{\text{CTX-M}}$ gene family in the isolates. However, the $bla_{\text{CTX-M}}$ gene family has been identified by others in *Salmonella enterica* and *E. coli* from poultry (Cavaco et al. 2008; Coque et al. 2008).

The five amplicons all from *K. oxytoca* corresponded to bla_{OXY} genes. In fact, in this wild-type species, this gene is chromosomally located and constitutively expressed at low levels, which usually confers low-level resistance to amino-penicillins but no significant resistance to other β-lactams (Livermore 1995; Fevre et al. 2005). This may explain the resistance phenotype obtained in this study only for AMP, an amino-penicillin (data not shown). However, the resistance patterns of *K. oxytoca* isolates are not in agreement with results of other authors, who refer the OXY hyperproduction associated with $bla_{\text{OXY-2}}$-encoded enzymes, caused by point mutations within the promoter sequence (Fournier et al. 1995), frequently considered false-positive ESBLs (Schmitt et al. 2007).

Antimicrobial susceptibility testing

The comparison of the resistant phenotype patterns of donor and respective recipient cells enabled us to hypothesize the presence of resistance plasmid genes that were transferred in the transconjugation assays (Tables 3, 4). The determination of antimicrobial susceptibility and resistance for the 13 antimicrobial agents revealed among the transconjugant cheese isolates a higher resistance in the β-lactam groups AMC (90%) and FOX (90%) and in the non-β-lactam group TET (65%), SXT (40%), IPM and MEM (10%). Antibiotics GEN and CIP were totally efficient showing 0% resistance (Table 3). These results are in agreement with the resistance phenotypes of the isolates recovered after a survey performed in Portuguese cheese (Amador et al. 2009). A different antibiotic ranking was found among transconjugant delicatessen meat isolates, in the non-β-lactam group TET (85%), SXT (50%), followed by IPM and MEM (30%) and in the β-lactam group FOX (45%) and AMC (20%) (Table 4).

When comparing the resistance phenotypes of donors and recipient strains, 0.5% (1/20) of the cheese isolates and 55% (11/20) of the delicatessen meat isolates showed total identity, which indicates the transference of these resistance patterns (Tables 3, 4). The resistance phenotypic transference among cheese isolates mismatch in average for two or three antibiotics, whereas just one antibiotic failed to be transferred among the meat isolates. These differences might be due to a higher antibiotic selective pressure in meat animals compared with dairy animals. Consequently, the microorganisms present in dairy products might have developed less efficient gene transfer mechanisms. In the meat animals group, antibiotics might be used throughout their lives, except for the withdrawal

Table 2 Characterization of the donor isolates

Reference	Identification[a]	RTE food source	Origin
Cheese			
37Q	*Proteus vulgaris*	Ewe cheese (semi-soft)—Seia	Market 2
138Q	*Serratia rubidaea*	Ewe cheese (ripened)—DOP-Évora	Supermarket A
39Q	*Enterobacter cloacae*	Ewe cheese (ripened)—DOP-Nisa	Supermarket A
41Q	*E. coli*	Ewe cheese (ripened)—Arraiolos	Supermarket A
137Q	*Pseudomonas aeruginosa*		
43Q	*E. coli*	Ewe cheese (ripened)—Arraiolos	Supermarket B
53Q	*Citrobacter freudii*		
45Q	*Morganella morganii*	Ewe cheese (soft)—Arraiolos	Supermarket A
47Q	*E. coli*	Mixture (cow, ewe and goat)—Covilhã	Supermarket B
55Q	*Proteus vulgaris* group	Goat cheese (ripened)—Castelo Branco	Market 1
57Q	*Proteus vulgaris* group	Goat cheese (ripened)—Carregal do Sal	Market 1
49Q	*Citrobacter braakii*	Ewe cheese (ripened)—V. Velha de Rodão	Market 1
68Q	*Enterobacter cloacae*		
51Q	*Enterobacter cloacae*	Ewe cheese—V. Velha de Rodão	Supermarket B
69Q	*E. coli*	Ewe cheese (semi-soft)—Castelo Branco	Market 1
59Q	*Enterobacter cloacae*		
61Q	*E. coli*	Goat cheese (ripened)—Povoa de Lanhoso	Supermarket A
63Q	*E. coli*		
65Q	*E. coli*		
71Q	*Shigella boydii*		
Delicatessen meat[b]			
134C	*Enterobacter amnigenus*	Ham	Butcher 1
25C	*E. coli*	Smoked sausage	Butcher 1
27C	*E. coli*		
72C	*E. coli*		
133C	*Pseudomonas fluorescens*		
4C	*E. vulneris*	Salted ribs	Butcher 1
29C	*E. coli*		
30C	*E. vulneris*		
31C	*E. coli*		
32C	*E. coli*		
33C	*E. coli*		
34C	*E. coli*		
2C	*E. vulneris*	Smoked sausage	Butcher 2
6C	*E. coli*		
7C	*E. coli*	Cold cuts	Supermarket A
21C	NI	Smoked and salted ham	Market 1
131C	*Enterobacter cloacae*	Artisanal meat salad	Supermarket A
130C	*Morganella morganii*		
23C	*Morganella morganii*	Boneless smoked and salted ham	Supermarket
73C	*Enterobacter amnigenus*		

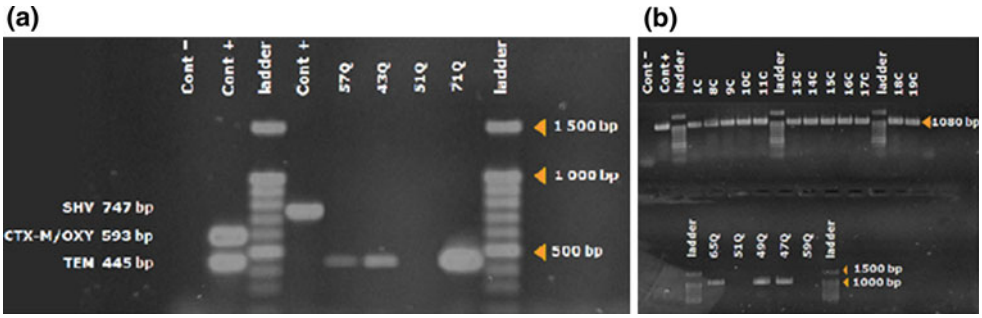

Fig. 1 Amplicons obtained by multiplex-PCR for detection of β-lactamase genes (bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}/bla_{OXY}$) of cheese donors isolates (**a**) and representative PCR for identification of bla_{TEM} genes of cheese donors and delicatessen meat transconjugants isolates (**b**). Negative control (Cont−), positive control (Cont+) and molecular marker 100 bp plus 1.5 Kb (ladder)

period previous to slaughter, whereas larger restrictions are imposed to antibiotics used in dairy animals, throughout the milking period.

TET resistance was frequently transferred both in meat isolates (12/17) and in cheese isolates (11/13). In the latter group of isolates, resistance phenotype to CHL and CIP were not transferred, though the resistance phenotype to AMC, FOX and ATM despite having been transferred in some cases, the majority failed to transfer. In contrast to meat isolates, the resistance phenotypes to AMC, FOX, SXT and CARB were transferred in most of the isolates (Tables 3, 4).

Sequence analysis of bla_{TEM}

Aiming at sequencing the bla_{TEM} genes previously detected, 47 donor and transconjugant isolates were selected for PCR amplification with primers TEM-F and TEM-R (Weill et al. 2004). The 1,080 bp amplicon corresponds to the coding region (861 bp) plus 214 bp upstream and 5 bp downstream of this region (Fig. 1b).

Consensus sequences obtained from reverse and forward sequence data of each isolate were 100% identical. The sequences yielded from this study were deposited in the EMBL/GenBank/DDBJ database (Tables 3, 4). The percentage of similarity between all possible sequence pairs of complete bla_{TEM} sequences varied between 99 and 100% (data not shown). All pairs of donor and respective transconjugant sequences were identical, which proved the in vitro transference of plasmid encoded bla_{TEM} genes from RTE enterobacteria to the recipient *E. coli*.

Among the 47 nucleotidic sequences produced in the present study, 43 bla_{TEM} sequences proved to be identical to three sequences deposited in the EMBL/GenBank/DDBJ database. The sequences obtained from 35 isolates were 100% similar to the TEM-1 sequence EF035622, two sequences were identical to the TEM-1 sequence DQ849328 and six sequences were identical to the TEM-1 sequence EF035581. Conversely, the sequences obtained from isolates 68Q and 22C were different from any other known sequence in databases (Tables 3, 4). The partial sequence of isolate 4C (49%—418 bp/861 bp) was 100% similar to the TEM-1 sequence EF035581, whereas the partial sequence of isolate 37Q (81%—697 bp/861 bp), except for a thymine residue in position 281 of the alignment, was identical to the TEM-1 sequence EF035622 (data not shown).

Although the RTE products had been produced at different places and sold at different commercial sites, no diversity was detected among the bla_{TEM} gene family, which evidences the great spread of $bla_{TEM\text{-}1}$ in these RTE types of food. Unlike the results obtained in this study, Hammad et al. (2009) found diversity in β-lactamases from ampicillin-resistant enterobacterial isolates obtained from traditional Egyptian Domiati cheese. In addition to the high prevalence of different types of SHV family, these isolates also contained CTX-M-14, OXY-1, OXA-1, and CMY-4. Nevertheless, those authors showed that the most frequent mechanism of ampicillin resistance was from a TEM-1-type β-lactamase.

Our results agree with those obtained by Sow et al. (2007), when analysing 25 multidrug-resistant strains of *Salmonella enteritidis* from human origin. All strains were resistant to at least one antibiotic including ampicillin, and all of them contained $bla_{TEM\text{-}1}$. Likewise, Briñas et al. (2002) found different variants of $bla_{TEM\text{-}1}$ and inhibitor-resistant bla_{TEM} (ITR) in ampicillin-resistant *E. coli* isolated from animal foods and feces of healthy humans and animals in Spain. In Denmark, Olesen et al. (2004) detected three different variants of $bla_{TEM\text{-}1}$, [of which $bla_{TEM\text{-}1b}$ was the most frequently detected, followed by $bla_{TEM\text{-}1a}$ and $bla_{TEM\text{-}1c}$] in ampicillin-resistant isolates (*E. coli* and *Salmonella* spp.) obtained from healthy and diseased food animals, whereas only a few isolates expressed $bla_{TEM\text{-}30}$, bla_{OXA} or bla_{PSE} β-lactamases. In France, Decré et al. (2004) referred $bla_{TEM\text{-}1}$ as the most common (1,458 out of 1,459 isolates) among Enterobacteriaceae collected from rectal swabs of hospitalized humans. In Germany, Schmitt et al. (2007) detected

Table 3 Comparison of resistance phenotypes to major antimicrobial classes between donor and transconjugants isolates from cheese, presence of β-lactamase genes in transconjugants and accession numbers of β-lactamase genes identified

Recipient	Donor species (reference)/ transconjugant (reference)	Resistance phenotype	bla_{TEM}[a]	Accession number/ TEM-type
E. coli J53	*Proteus vulgaris* (37Q)	AMP, AMC, FOX, CHL, TET		GQ983321/TEM-1
	Transconjugant (38Q)	AMP, TET	+	
	E. coli (41Q)	AMP, SXT, TET		GQ983324/TEM-1
	Transconjugant (42Q)	AMP	+	GQ983325/TEM-1
	E. coli (43Q)	AMP, AMC, FOX, SXT, TET		GQ983326/TEM-1
	Transconjugant (44Q)	AMP, SXT, TET	+	GQ983327/TEM-1
	Morganella morganii (45Q)	AMP, AMC, FOX, CHL, TET		
	Transconjugant (46Q)	AMP, FOX, TET	+	
	Citrobacter braakii (49Q)	AMP, AMC, FOX, SXT, TET		GQ983330/TEM-1
	Transconjugant (50Q)	AMP, SXT, TET	+	GQ983331/TEM-1
	Enterobacter cloacae (51Q)	AMP, AMC, FOX, CEPH3, ATM		
	Transconjugant (52Q)	AMP, FOX	−	
	Citrobacter freudii (53Q)	AMP, AMC, FOX, CEPH3, ATM, CHL, SXT		GQ983332/TEM-1
	Transconjugant (54Q)	AMP, AMC, FOX, ATM, SXT	+	GQ983333/TEM-1
	Proteus vulgaris (55Q)	AMP, AMC, FOX, CEPH3, ATM, CHL, SXT		
	Transconjugant (56Q)	AMP, AMC, FOX, ATM, SXT	+	
	Enterobacter cloacae (59Q)	AMP, AMC, FOX, CEPH3, CPO, ATM, CARB		
	Transconjugant (60Q)	AMP, AMC, CPO, CARB	+	
	Enterobacter cloacae (68Q)	AMP, AMC, FOX, CEPH3, CPO, ATM		GQ983341/TEM-180
	Transconjugant (144Q)	AMP, AMC, FOX, CEPH3, CPO	+	
	P. aeruginosa (137Q)	AMP, AMC, FOX, CEPH3, CHL, SXT, TET		
	Transconjugant (143Q)	AMP, AMC, FOX, SXT, TET	−	
	K. oxytoca (138Q)	AMP, AMC, FOX, CEPH3, CPO, ATM, CHL, CIP, TET		
	Transconjugant (145Q)	AMP, AMC, FOX, CEPH3, CPO, TET	−	
E. coli HB101	*Enterobacter cloacae* (39Q)	AMP		GQ983322/TEM-1
	Transconjugant (40Q)	AMP	+	GQ983323/TEM-1
	E. coli (47Q)	AMP, AMC, FOX, CEPH3, TET		GQ983328/TEM-1
	Transconjugant (48Q)	AMP, TET	+	GQ983329/TEM-1
	Proteus vulgaris (57Q)	AMP, AMC, FOX, CEPH3, CPO, CARB, TET		
	Transconjugant (58Q)	AMP, AMC, FOX, CEPH3, CARB	+	
	E. coli (61Q)	AMP, AMC, FOX, CEPH3, ATM, TET		GQ983334/TEM-1
	Transconjugant (62Q)	AMP, TET	+	GQ983335/TEM-1
	E. coli (63Q)	AMP, AMC, FOX, SXT, TET		GQ983336/TEM-1
	Transconjugant (64Q)	AMP, TET	+	GQ983337/TEM-1
	E. coli (65Q)	AMP, AMC, FOX, TET		GQ983338/TEM-1
	Transconjugant (66Q)	AMP, TET	+	GQ983339/TEM-1
	E. coli (69Q)	AMP, AMC, FOX		GQ983342/TEM-1
	Transconjugant (70Q)	AMP	+	GQ983343/TEM-1
	Shigella boydii (71Q)	AMP, AMC, FOX, CEPH3, ATM, SXT, TET		GQ983344/TEM-1
	Transconjugant (67Q)	AMP, TET	+	GQ983340/TEM-1

[a] TEM presence in transconjugants; underlined—β-lactam antibiotic

Ampicillin (AMP); Amoxicillin/clavulanic acid combination (AMC); cefoxitin (FOX); ceftazidime and/or cefotaxime (CEPH3); cefpirome (CPO); aztreonam (ATM); carbapenems, imipenem and/or meropenem (CARB); chloramphenicol (CHL); ciprofloxacin (CIP); trimethoprim/sulfamethoxazol (SXT) and tetracycline (TET)

bla_{TEM-1} and bla_{OXY} in *K. pneumoniae*, *K. oxytoca* and *E. coli* isolated from hospital patients. In The Netherlands, Vo et al. (2007) found bla_{TEM-1} in enterobacteria isolated from horse clinical samples. In Hong-Kong, Ho et al. (2008) detected the presence of bla_{TEM-1b} and bla_{TEM-1c} in isolates of human feces.

Table 4 Comparison of resistance phenotypes to major antimicrobial classes between donor and transconjugants isolates from cheese, presence of β-lactamase genes in transconjugants and accession numbers of β-lactamase genes identified

Recipient	Donor species (reference)/ transconjugant (reference)	Resistance phenotype	bla_{TEM}[a]	Accession number/ TEM-type
E. coli J53	*E. vulneris* (2C)	AMP, SXT, TET		
	Transconjugant (19C)	AMP, SXT, TET	+	GQ983312/TEM-1
	E. vulneris (4C)	AMP, TET		GQ983347/TEM-1
	Transconjugant (16C)	AMP	+	GQ983309/TEM-1
	E. coli (6C)	AMP, FOX, SXT, TET		
	Transconjugant (9C)	AMP, SXT, TET	+	GQ983302/TEM-1
	E. coli (7C)	AMP, FOX, ATM, SXT, TET		
	Transconjugant (20C)	AMP, FOX, ATM, SXT, TET	+	GQ983313/TEM-1
	E. coli (25C)	AMP, SXT, TET		
	Transconjugant (11C)	AMP, SXT, TET	+	GQ983304/TEM-1
	E. coli (27C)	AMP, ATM, SXT, TET		GQ983317/TEM-1
	Transconjugant (13C)	AMP, ATM, SXT	+	GQ983306/TEM-1
	E. vulneris (30C)	AMP, TET		
	Transconjugant (8C)	AMP	+	GQ983301/TEM-1
	E. coli (31C)	AMP, SXT, TET		
	Transconjugant (18C)	AMP,TET	+	GQ983311/TEM-1
	E. coli (32C)	AMP, SXT, TET		
	Transconjugant (14C)	AMP, SXT, TET	+	GQ983307/TEM-1
	E. coli (33C)	AMP, SXT, TET		GQ983319/TEM-1
	Transconjugant (17C)	AMP, SXT, TET	+	GQ983310/TEM-1
	E. coli (72C)	AMP		GQ983345/TEM-1
	Transconjugant (12C)	AMP	+	GQ983305/TEM-1
	Enterobacter amnigenus (134C)	AMP, TET		
	Transconjugant (10C)	AMP	+	GQ983303/TEM-1
	Enterobacter cloacae (130C)	AMP, AMC, FOX, CARB		
	Transconjugant (147C)	AMP, FOX, CARB	−	
E. coli HB101	*E. coli* (29C)	AMP, FOX, TET		GQ983318/TEM-1
	Transconjugant (1C)	AMP, FOX, TET	+	GQ983346/TEM-1
	E. coli (34C)	AMP, SXT, TET		GQ983320/TEM-1
	Transconjugant (15C)	AMP, SXT, TET	+	GQ983308/TEM-1
	NI (132C)	AMP, AMC, FOX, CEPH3, ATM, CARB, TET		
	Transconjugant (21C)	AMP, AMC, FOX, CEPH3, ATM, TET	+	GQ983314/TEM-1
	Morganella morganii (131C)	AMP, FOX, ATM, CARB,TET		
	Transconjugant (148C)	AMP, FOX, ATM, CARB,TET	−	
	Pseudomonas fluorescens (133C)	AMP, AMC, FOX, CPO, ATM, CARB, SXT, TET		
	Transconjugant (149C)	AMP, AMC, FOX, CPO, ATM, CARB, SXT	−	
	Enterobacter amnigenus (73C)	AMP, AMC, FOX, CEPH3, CPO, CARB		
	Transconjugant (22C)	AMP, AMC, FOX, CEPH3, CPO, CARB	+	GQ983315/TEM-179
	Morganella morganii (23C)	AMP, FOX, ATM, CARB, TET		
	Transconjugant (146C)	AMP, FOX, ATM, CARB, TET	+	

[a] TEM presence in transconjugants; underlined—β-lactam antibiotic

Ampicillin (AMP); Amoxicillin/clavulanic acid combination (AMC); cefoxitin (FOX); ceftazidime and/or cefotaxime (CEPH3); cefpirome (CPO); aztreonam (ATM); carbapenems, imipenem and/or meropenem (CARB); chloramphenicol (CHL); ciprofloxacin (CIP); trimethoprim/sulfamethoxazol (SXT) and tetracycline (TET)

The deduced bla_{TEM} amino acid sequences obtained from the 43 isolates represented by isolate 15C are 100% similar with the identified sequences TEM-1 (EF035622, EF035581, EF035587, EF035579, EF035580, EF035588, AF309824, 1d-AF42713, 1d-AF18820 and 1e-AF42713) (Fig. 2). Eight sequences carry silent mutations, as

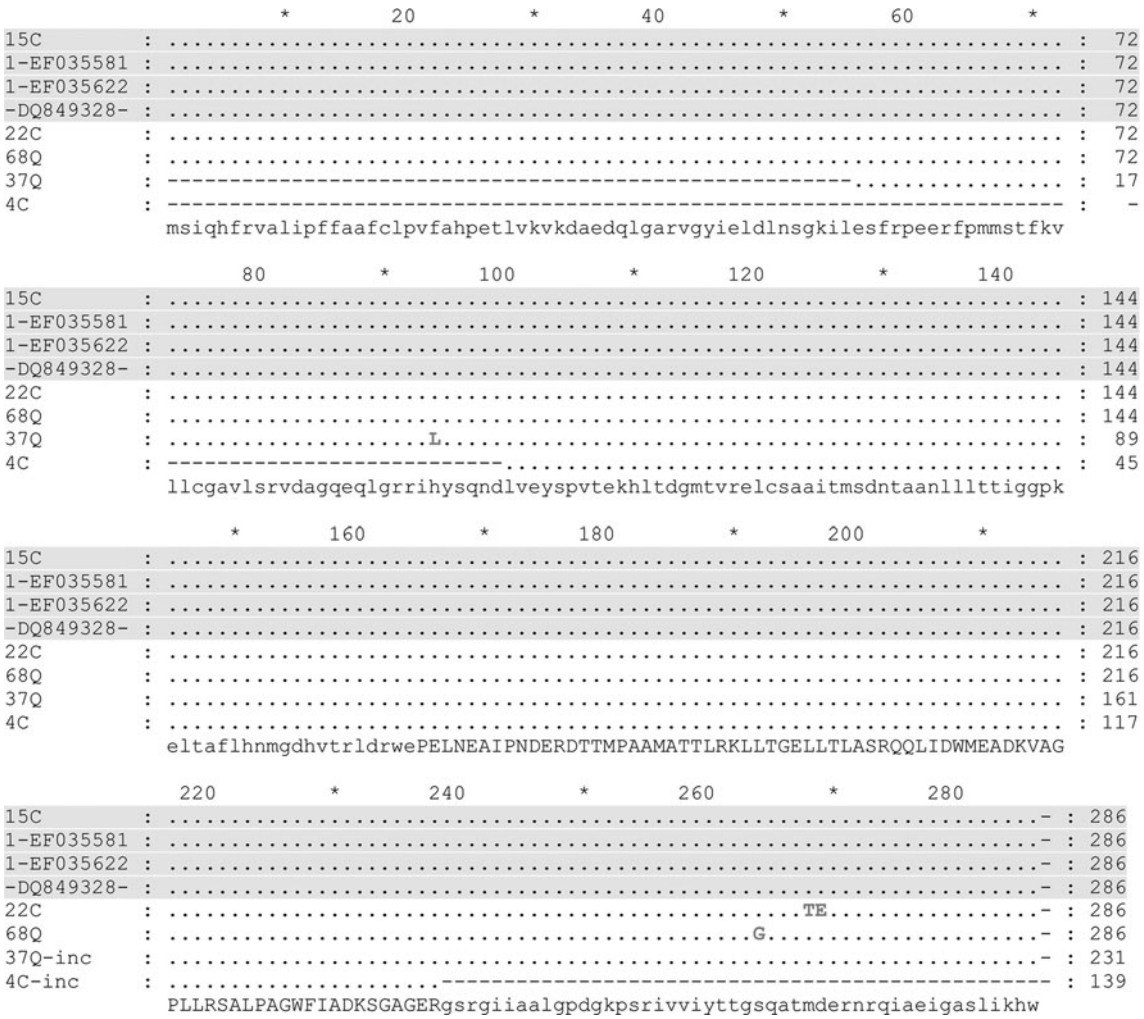

Fig. 2 Amino acid sequence alignment of the *bla*$_{TEM}$ gene/coding region, obtained from 47 isolates (Tables 3, 4) and three sequences retrieved from EMBL/GenBank/DDBJ database (1-EF035581, 1-EF035622. Hyphens represent gaps included in the sequence. Sequence 15C stands for sequences 40Q, 41Q, 42Q, 43Q, 44Q, 47Q, 48Q, 49Q, 50Q, 53Q, 54Q, 61Q, 62Q, 63Q, 64Q, 65Q, 66Q, 67Q, 69Q, 70Q, 71Q, 1C, 8C, 9C, 10C, 11C, 12C, 13C, 14C, 16C, 17C, 18C, 19C, 20C, 21C, 24C, 27C, 29C, 33C, 34C, 39Q and 72C, which are identical. Sequences 22C and 68Q are different from any other known sequence on databases

commonly referred by several authors. Conversely, the sequences obtained from isolates 22C and 68Q are different from any other known published sequence, which indicates two new enzymes of the TEM family group. In fact, here we describe for the first time, two new TEM enzymes being proposed as TEM-179 and TEM-180, respectively (Tables 3, 4). Two non-conserved substitutions S264G (S268G Ambler position) (Ambler et al. 1991) in sequence from isolate 68Q and M268T (M272T Ambler position) in sequence from isolate 22C were found. Moreover, a conserved amino acid substitution D269E (D273E Ambler position) in sequence from isolate 22C was also observed (Fig. 2). Many ESBLs are generated by mutations in genes coding for broad-spectrum enzymes, and carry multiple point mutations in the coding and promoter regions. Some of the substitutions are silent and others cause amino acid changes which affect the enzyme activity. These changes are clustered around seven evolutionarily conserved sequence boxes (Caniça et al. 1996). Thus, in strains 22C and 68Q there is the theoretical possibility of conformational changes in the protein and therefore an enzymatic functional alteration, more or less efficient to a certain antibiotic. This will be further investigated. The partial amino acid sequence from isolate 4C was identical to the TEM-1 sequences, but the partial sequence from isolate 37Q differed from the former group by a non-conserved substitution H94L (H96L Ambler position) (Fig. 2).

Conclusions

In summary, about 31.3% of β-lactam resistant isolates collected from Portuguese cheeses and delicatessen meats proved to transfer resistance determinants in transconjugation assays with *E. coli*. Most of them belong to the Enterobacteriaceae family, *E. coli* being the most prevalent (42.5%) species.

bla_{TEM} genes sequences were present in 85% of these isolates and were identical in the respective transconjugants, proving the ability of conjugative plasmids encoding bla_{TEM} to be transferred in vitro from RTE microorganisms to *E. coli*. Although the food products were from different regions in Portugal, no diversity was detected among bla_{TEM} gene family, suggesting its great spread in these type of foods. Nevertheless, two isolates presented two new enzymes, TEM-179 and TEM-180. The phenotypes of these isolates cannot be explained merely by the presence of bla_{TEM}, and further studies will screen for other genes such other ESBL, AmpC β-lactamases and eventually other broad-spectrum β-lactamases.

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