First detection of OKP-A β-lactamase in two *Serratia marcescens* isolates in China

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SUMMARY

Two strains of *Enterobacteriaceae* producing prodigiosin were isolated from meat in the Sichuan province of China in 2010. The strains were identified by Vitek system, 16S rDNA, *rpoB*, *pfs* and *luxS* genes. Minimum inhibitory concentrations were determined using the broth microdilution method. The two strains were screened for the presence of β-lactamase genes (bla_{TEM}, bla_{SHV}, bla_{OKP}, and bla_{CTX-M} genes). Based on PCR amplification and 16S rDNA sequencing the analysed strains were identified as *Serratia marcescens*. In addition, morphological and biochemical identification showed that the two stains were definitely *S. marcesens*. Antimicrobial susceptibility test showed that both strains were resistant to ampicillin and first-generation cephalosporins while being susceptible to cefotaxime, ceftiofur, ceftriaxone, imipenem and aztreonam. It was found that *bla*OKP had been identified first from the two *S. marcescens* strains, ch1 and ch2. The isolates were closely related as shown by pulsed-field gel electrophoresis (PFGE). The narrow-spectrum OKP-A β-lactamase gene *bla*OKP-A-13 was found to be chromosomally located in *S. marcescens*. The isolates produced a β-lactamase with a pI of approximately 8.2, which corresponds to the OKP-A family. Findings indicate that OKP enzymes are not *Klebsiella pneumoniae*-specific chromosomal β-lactamases, and the first isolation of *S. marcescens* producing OKP-A β-lactamase suggests that the *bla*OKP gene may be disseminated between different species.

KEY WORDS: Detection, OKP-A β-lactamase, *Serratia marcescens*, China

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INTRODUCTION

The chromosomally encoded bla_{OKP} enzymes are grouped in a single new family of Other *Klebsiella pneumonia* β-lactamases, which belongs to molecular class A of Ambler (Hæggman *et al.*, 2004; Siebor *et al.,* 2005). *K. pneumoniae* isolates may be naturally resistant to ampicillin, amoxicillin and ticarcillin but not to extended-spectrum β-lactams because of a constitutively expressed chromosomal β-lactamase, including the bla_{SHV}, bla_{LEN}, and bla_{OKP} (Hæggman *et al.*, 2004; Mendonça *et al.,* 2009). Studies have shown that the bla_{OKP} gene diversified into many variants, which can be classified into two subgroups based on nucleotide sequences: the bla_{OKP-A} and bla_{OKP-B} β-lactamases.

The bla_{OKP} chromosomal β-lactamase gene has been used as a phylogenetic marker in *K. pneumoniae* in case of its low level of nucleotide divergence and evolved in parallel with the *K. pneumoniae* phylogenetic group KpII (Fevre *et al.,*2005; Hæggman *et al.,* 2004).

To date, there have been no reports on the distribution of the bla_{OKP} gene among *Serratia marcescens* strains. The spread of β-lactamases among members of the family *Enterobacteriaceae* has been increasingly observed throughout the world (Queenan *et al.,* 2006). *S. marcescens* is an opportunistic pathogen associated with respira-

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tory tract infections, urinary tract infections, septicemia, meningitis, and wound infections. Moreover, it has been shown to be resistant to a wide variety of antimicrobial agents (Begic *et al.,* 2008; Chen *et al.,* 2003; Thompson *et al.,* 2007). The emergence of extended-spectrum β-lactamases (ESBL)-exhibiting *S. marcescens* causing nosocomial infection represents serious problem worldwide (Ivanova *et al.,* 2008; Po-An *et al.,* 2010). Moreover, *S. marcescens* could cause Cucurbit yellow vine disease (CYVD) in plants such as squash, pumpkin, watermelon, rice and cotton (Zhang *et al.,* 2005). Besides, laboratory contamination was observed due to *S. marcescens* as a cause of a pseudo-outbreak reported by Dundar *et al.* (2009).

Here we describe the bla_{OKP-A} gene found in *S. marcescens* strains, isolated from meat in Sichuan, China. The two strains producing narrow spectrum β-lactamase OKP-A-13 are resistant to ampicillin and cephalosporins.

MATERIALS AND METHODS

Bacteria strains and identification

During the study carried out from 2009 to 2010 to demonstrate the β-lactam-resistance phenotypes and genotypes of *Enterobacteriaceae* strains isolated from food samples, two *S. marcescens* stains were collected from meat in Sichuan, China. The two strains will be referred to as strain ch1 and ch2 throughout the paper. Identification of the two strains to the species level was performed by Vitek system (bioMerieux, Nurtingen, Germany).

Genomic DNA for PCR amplification was prepared with a TIANamp Bacteria DNA Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. They were also confirmed using 16S rDNA sequences (Singh *et al.,* 2007; Zhu *et al.,* 2007) and *rpoB* gene (Mollet *et al.,* 1997) by PCR assay. In addition, the *S. marcescens* stains were examined for the presence of *pfs* and *luxS* genes (Hu *et al.,* 2008). The primers used to amplify 16S rDNA, *rpoB*, *pfs* and *luxS* genes are listed in Table 1. Each 50 l PCR mixture consisted of 1 l of template, 5 l 10×PCR Buffer, 1.5 mM $MgCl_2$, 200 M dNTP, 0.4 M primers and 2.5U TaqDNA polymeras. PCR for 16S rDNA and *rpoB* was performed in a DNA thermal cycler (Bio-Rad, Hercules, CA) using the following program: an initial denaturation step of 94°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C or 51°C for 1 min and extension at 72°C for 1.5 min or 1 min, followed by a final elongation at 72°C for 10 min. Amplified PCR products were analyzed on 0.8% (w/v) agarose gels. PCR amplification conditions for *pfs* and *luxS* were as follows: an initial denaturation step of 94°C for 5 min, 30 cycles of denaturation at 94°C for 45 s, annealing at 55°C or 51°C for 40s and extension at 72°C for 25 s, followed by a final elongation at 72°C for 10 min. Amplified PCR products were analyzed on 2.0% (w/v) agarose gels.

The PCR products were purified using TaKaRa Agarose Gel DNA Purification Kit Version 2.0 (TaKaRa, Dalian, China), cloned into the pUcm-T Vector (Sangon, Shanghai, China) using TaKaRa DNA Ligation Kit Version 2.1 (TaKaRa, Dalian, China), and transformed into *E. coli*

TABLE 1 - *PCR primers for S.marcescens strains identification.*

Gene	*Sequence(5'-3')*	*Tm*	*Extension time*	*PCR product size (bp)*
16S rDNA	AGAGTTTGATCCTGGCTCAG TACGGCTACCTTGTTACGACTT	50°C	1.5 min	1507
rpoB	AACCAGTTCCGCGTTGGCCTGG CCTGAACAACACGCTCGGA	55°C	1 min	1090
luxS	GCTGGAACACCTGTTCGC ATGTAGAAACCGGTGCGG	51°C	25s	102
pfs	CCGGCATCGGCAAAGTCT ATCTGGCCCGGCTCGTAGCC	55°C	25s	193

DH5 . Subsequently, the amplicons were sequenced by Shanghai Sangon Bioengineering Co., Ltd. The nucleotide sequences were analyzed with software (BLAST) available from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Confirmed isolates were stored in Trypticase soy broth containing 20% glycerol at -80°C until use.

MICs determination

The minimum inhibitory concentrations (MICs) of 19 antimicrobial agents alone or associated with β-lactamase inhibitors were determined by the broth microdilution method recommended by the Clinical and Laboratory Standards Institute (CLSI, 2009). Antimicrobials tested were ampicillin, ampicillin/sulbactam, ticarcillin, ticarcillin/ clavulanic acid, amoxicillin/clavulanic acid, cefoxitin, cephalexin, cefotaxime, ceftiofur, ceftriaxone, imipenem, aztreonam, gentamicin, amikacin, kanamycin, netilmicin, streptomycin, norfloxacin, and ciprofloxacin. *Escherichia coli* ATCC25922 and *E. coli* ATCC35218 were used for quality control.

Amplification of β-lactamase genes and sequencing

Different primers were used to amplify bla_{TEM}, bla_{SHV}, bla_{OKP}, and bla_{CTX-M} β-lactamase genes. The 861-bp bla_{OKP} gene was amplified by PCR using the primers OKP1F (TGGTTATGCGTTATATTCGCCTG) (positions 1 to 18 of the OKP-A-13 coding sequence; GenBank FJ534513) and OKP1R (GCTTAGCGTTGCCAGTGCT) (positions 844 to 861 of the OKP-A-13 coding sequence; GenBank FJ534513), and primers OKP2F (TCTTTACTCGCCTT TATCGGCC) and OKP2R (TTAGCG TTGCCAGTGCTCGATC) (Ribot *et al.*, 2006). Presence of bla_{TEM}, bla_{SHV}, and bla_{CTX-M} resistance genes were also checked by PCR using specific oligonucleotide primers as follows: TEM-F and TEM-R, specific for *bla* (Guo-Bao *et al.,* 2007); SHV-F and SHV-R, specific for *bla* (Melano *et al.,* 2006; Yagi *et al.,* 2000); and CTX-M-F and CTX-M-R, specific for *bla* (Edelstein *et al.,* 2003). Briefly, the bla_{OKP} gene was amplified on a DNA thermal cycler (Bio-Rad, Hercules, CA) with a final volume of 50 l consisting of 5 l of template, 5 µl 10×PCR Buffer, 1.5 mM $MgCl_2$, 200 M dNTP, 0.4 M each of the primers, and 2.5 U Taq DNA polymerase. The cycling conditions were as follows: an initial denaturation step of 94°C for 4 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 51°C for 1 min, and extension at 72°C for 50s, followed by a final elongation at 72°C for 10 min. PCR products were ligated to pUcm-T Vector (Sangon, Shanghai, China) using TaKaRa DNA Ligation Kit Version 2.1 (TaKaRa, Dalian, China) and transformed into *E. coli* DH5α. Subsequently, the PCR products were sequenced by the Shanghai Sangon Bioengineering Co., Ltd. The nucleotide and deduced protein sequences were compared with those available in GenBank and at www.lahey. org/studies/webt. html.

Plasmid analysis and transformation

Plasmid DNA was extracted from the strains using the TIANpure Midi Plasmid Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. The extracted plasmid DNA was digested with *Xba*I or *Hind*III. The plasmid DNA from *S. marcescens* ch1 and ch2 was electroporated into *Escherichia coli* DH10B according to the manufacturer's instructions (MicroPulser™, Bio-Rad) (Naas *et al.,* 2003). Elimination of plasmid DNA from strain ch1 and ch2 was as previously described (El-Mansi *et al.,* 2000). Plasmid DNA was also extracted from cured (plasmid eliminated) derivative isolates as above described. Electrophoresis was performed in a 0.8% agarose gel at 80 V for 2h, DL15,000 and λDNA/*Hind* III DNA (Tiangen, Beijing, China) were used as the molecular size marker.

PFGE

Pulsed-field gel electrophoresis (PFGE) was used to study the clonal relatedness of the strains. *Xba*I digested DNA fragments were analyzed by pulsefield gel electrophoresis (PFGE) using 1% agarose gels and a CHEF MAPPER electrophoresis system (Bio-Rad). The electrophoresis conditions were as previously described (Ribot *et al.*, 2006). After migration, the gel was stained in a solution of 0.5 µg/ml ethidium bromide for 30 min. Relatedness of the isolates was interpreted using the criteria established by Tenover *et al.* (Tenover *et al.,* 1995).

Isoelectric focusing

Crude β-lactamase extracts were prepared by ultrasonic treatment of bacterial cells. After re-

moval of whole cells and debris by centrifugation, the supernatant was used to determine the isoelectric point (pI). Electrophoresis was performed using precast polyacrylamide gels, pH 7 to 10 (Bio-Rad, Hercules, CA, USA). Enzyme activity was visualized by gel staining with Nitrocefin (Oxoid, Basingstoke, Hampshire, UK). pIs were determined after comparison with the know pI of β-lactamase SHV12 (pI of 8.2, GenBank FJ685654).

Nucleotide sequence accession number

The nucleotide sequences of the 16s rDNA, *bla*TEM-1, and *bla*OKP-A-13 genes reported in this work are available in the GenBank nucleotide database under the accession numbers GU991997, HM348775 and HM348776, respectively.

RESULTS AND DISCUSSION

Among all 536 strains recovered from meat in 2009 and 2010, two *S. marcescens* isolates producing prodigiosin were identified by Vitek system, 16S rDNA, *rpoB*, *pfs* and *luxS* genes (Figure 1). The identities of the PCR products also were confirmed by commercial sequencing. Morphological and biochemical identification, PCR amplification and 16S rDNA sequencing showed that these two stains were definitely *Serratia marcescens*. Antimicrobial susceptibility test showed that both strains were resistant to ampicillin, amoxicillin, cefoxitin, and cephalexin while being susceptible to cefotaxime, ceftiofur, ceftriaxone, imipenem, and aztreonam. The isolates were also tested for antimicrobial susceptibility by broth microdilution. The MICs of 19 antimicrobial agents determined by broth microdilution are shown in Table 2. The transformants exhibited lower MICs of ampicillin (ch1, 2.5 µg/ml; ch2, 5 µg/ml) and cefoxitin (ch1, 2.5 µg/ml; ch2, 5 µg/ml) compared to the parent strains.

Sequencing of the amplicons of both strains showed 100% sequence identity with the *bla*OKP-A-13. At present, this is the first report of bla_{OKP} gene in a food-borne *S. marcescens* strain. Furthermore, specific PCR and sequencing results revealed that both isolates harbored the bla_{TEM-1} gene. Primers specific for bla_{SHV} and bla_{CTX-M} genes gave negative results. OKP enzymes have been found only in *K. pneumoniae* isolates (Siebor *et al.,* 2005) prior to this study. To date, there are only a few studies on OKP chro-

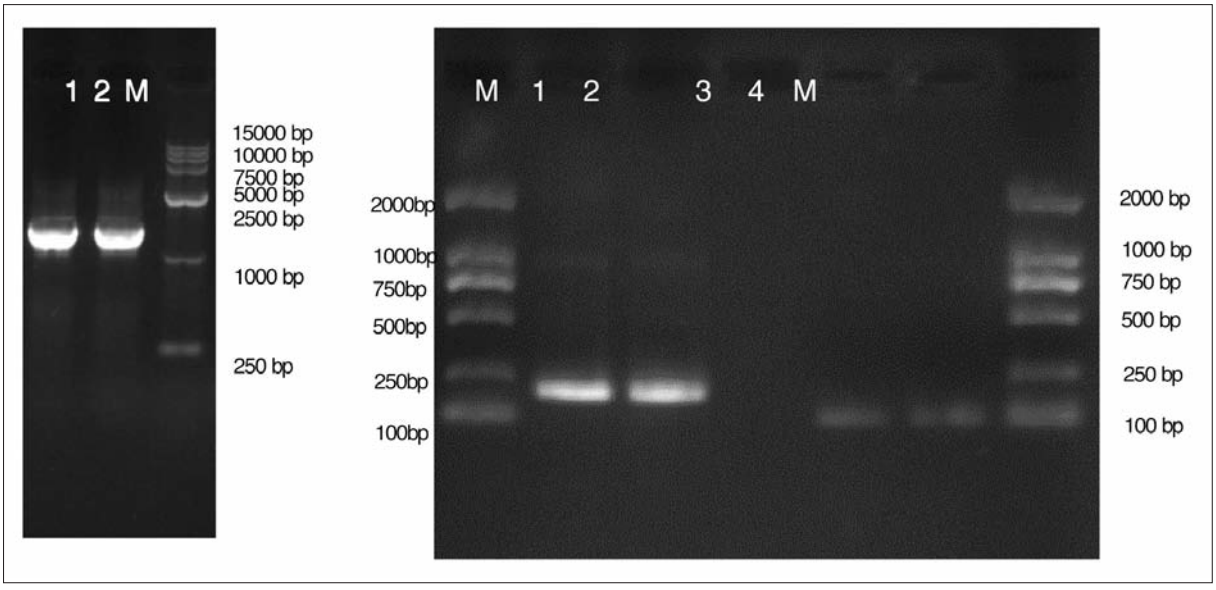

FIGURE 1 A - *PCR amplification of 16rDNA genes. Lane M: DL15,000 DNA marker (Tiangen, Beijing, China), lanes 1 and 2: S. marcescens strains ch1 and ch2, respectively.* B - *PCR amplification of pfs and luxS genes. Lane M: DL2,000 DNA marker (Tiangen, Beijing, China), lanes 1 and 2: pfs detected in S. marcescens strains ch1 and ch2, respectively. lanes 3 and 4: luxS detected in S. marcescens strains ch1 and ch2, respectively.*

mosomal β-lactamase genes in *K. pneumonia*. Majority of OKP β-lactamase-producing isolates have been found *et al.,* in Europe and Canada, and focuses on human blood, and respiratory and urinary tracts (Fevre 2005; Hæggman *et al.,* 2004; 10, Mendonça *et al.,* 2009; Siebor *et al.,* 2005). A previous study had discovered four new bla_{OKP} genes encoding for OKP-A-13 (FJ534513), -14 (FJ534512), -15 (FJ755841), and -16 (FJ755840) in *K. pneumoniae* isolates in China in 2009, and the diversity of these OKP β-lactamase genes has been confirmed by phylogenetic analysis (Li-Kou *et al.,* 2011).

TABLE 2 - *MICs of 19 antimicrobial agents for two S. marcescens isolates.*

	MIC (μg/ml)	
Antimicrobial agent(s)	*S. marcescens ch 1/transformants*	*S. marcescens ch 2/transformants*
Ampicillin	≥32/2.5	≥32/5
Ampicillin/sulbactam	4	4
Ticarcillin	2	4
Ticarcillin/clavulanic acid	≤0.5	≤0.5
Amoxicillin/clavulanic acid	2	2
Cefoxitin	≥32/2.5	≥32/5
Cephalexin	≥32	≥32
Cefotaxine	≤1	2
Ceftiofur	1	2
Ceftriaxone	≤0.5	≤0.5
Imipenem	≤0.5	≤0.5
Aztreonam	≤0.5	≤0.5
Gentamicin	≤0.5	≤0.5
Amikacin	≤1	≤1
Kanamycin	2	2
Netilmicin	≤1	≤1
Streptomycin	2	2
Norfloxacin	≤0.5	≤0.5
Ciprofloxacin	≤0.5	≤0.5

Results show that ch1 and ch2 contained plasmids of approximately 18 kb (Figure 2). Digestion with *Hind* III endonuclease yielded seven bands of approximately 0.15 to 7.5 kb (Figure 3). The plasmids have identical banding patterns. PCR amplification of plasmid DNA extracts from both parent and transformant strains were bla_{TEM}-positive and bla_{OKP}-negative. Plasmid DNA from two *S. marcescens* cured (plasmid eliminated) derivative strains was extracted as described above and the presence or absence of plasmid DNA was tested using agarose gel electrophoresis. Clearly, as can be seen from Figure 4, the *S. marcescens* strains appeared to have completely lost their indigenous plasmid. Subsequently, PCR amplification of bla_{TEM} and bla_{OKP} genes in two *S. marcescens* cured derivative strains total DNA was bla_{TEM}-negative and bla_{OKP}-positive (Figure

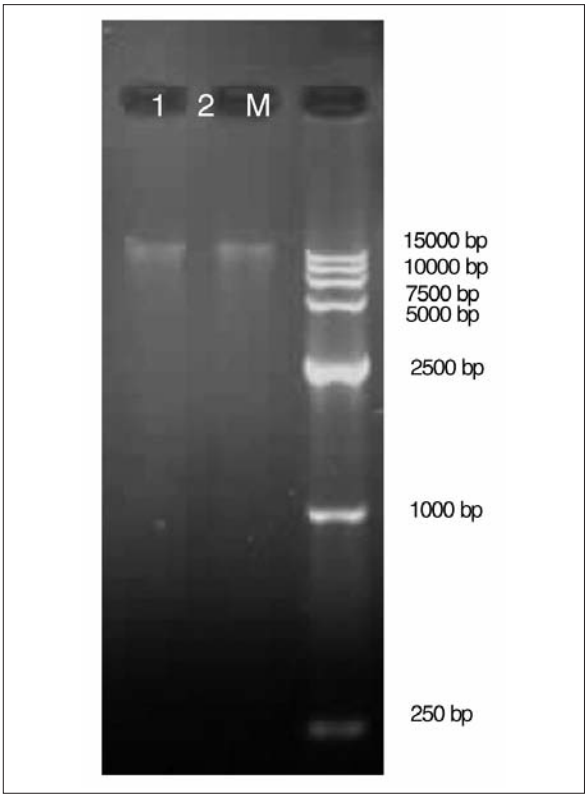

FIGURE 2 - *Plasmid of S. marcescens isolates. Lane M: DL15,000 DNA marker (Tiangen, Beijing, China), lanes 1 and 2: S. marcescens strains ch1 and ch2, respectively.*

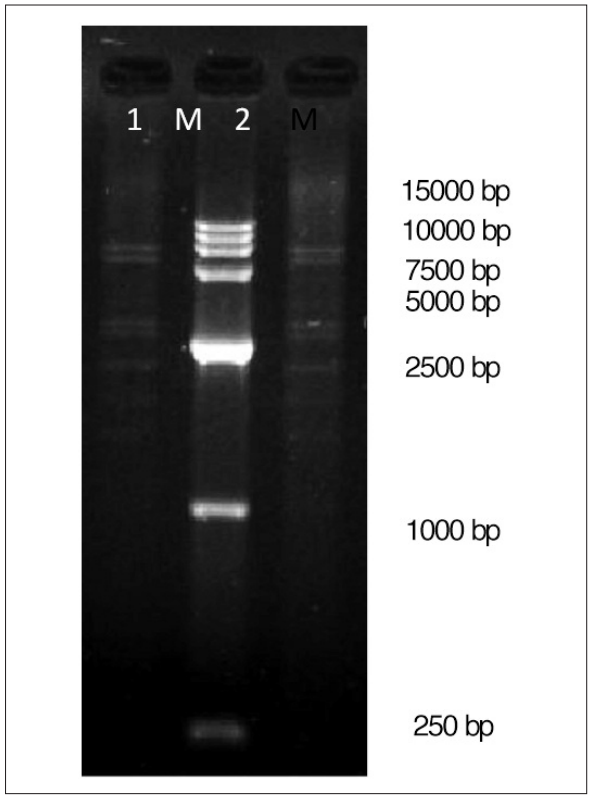

FIGURE 3 - *Restriction patterns of HindIII -digested plasmid DNA fragments of transformants. Lane M: DL15,000 DNA marker (Tiangen, Beijing, China), lanes 1 and 2: S. marcescens strains ch1 and ch2 transformants, respectively.*

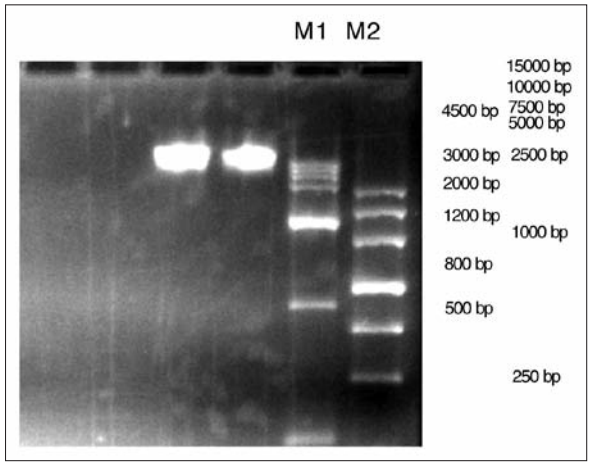

FIGURE 4 - *Plasmid of S. marcescens and its cured derivative isolates. Lane M1: DNA marker III, Lane M2: DL15,000 DNA marker (Tiangen, Beijing, China), lanes 1 and 2: S. marcescens strains ch1 and ch2, respectively. Lanes 3 and 4: S. marcescens strains ch1 and ch2 derivative isolates, respectively.*

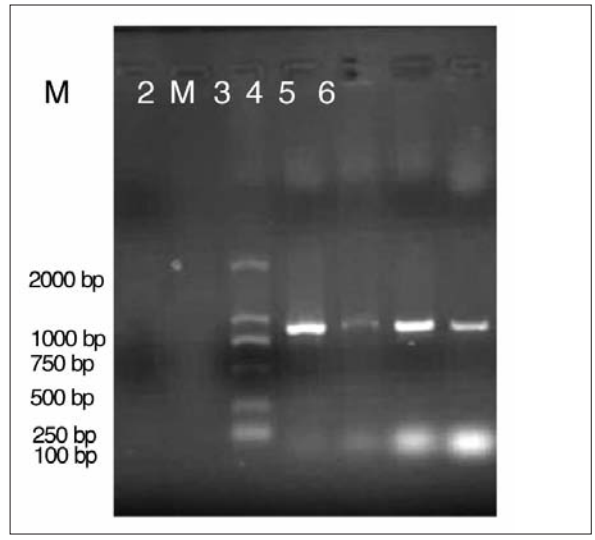

FIGURE 5 - *PCR amplification of blaTEM and bla_{OKP} genes in S. marcescens derivative isolates. Lane M: DL2,000 DNA marker (Tiangen, Beijing, China), lanes 1 and 2: bla_{TEM} negative in S. marcescens strains ch1 and ch2 derivative isolates, respectively. Lanes 3 and 4: bla_{OKP} positive in S. marcescens strain ch1 derivative isolates. Lanes 5 and 6: bla_{OKP} positive in S. marcescens strain ch2 derivative isolates.*

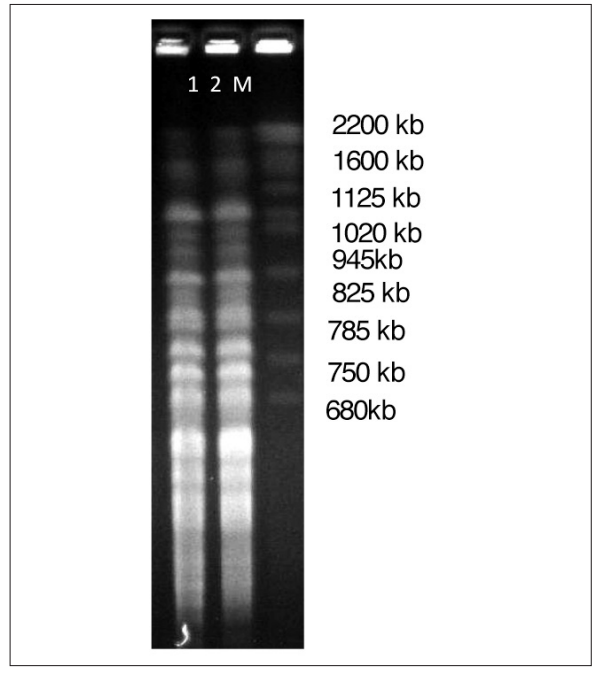

FIGURE 6 - *PFGE patterns of S. marcescens isolates after digestion with XbaI. Lane M: CHEF DNA Size Marker (Saccharomyces cerevisiae) (Bio-Rad, Hercules, CA), lanes 1 and 2: S. marcescens strains ch1 and ch2, respectively.*

5). However, as described above, it was bla_{TEM}-positive and bla_{OKP}-negative for PCR in both parent and transformant strains' plasmid DNA extracts. Elimination of plasmid DNA, specific PCR analysis of plasmids and transformant experiments failed to produce transformants containing OKP enzymes implies that the bla_{OKP} gene encoding for these β-lactamases is present in the chromosome of *S. marcescens* and that the bla_{OKP} antibiotic resistance gene may exist in other species. Likewise, SHV β-lactamase which had a narrow spectrum of activity was first described as a chromosomally encoded β-lactamase in *K. pneumoniae* isolates, then it evolved and was found in other *Enterobacteriaceae* species (Heritage *et al.,* 1999)

PFGE patterns of the two OKP β-lactamase-producing *S. marcescens* isolates are shown in Figure 6. Interestingly, results show that the two strains behave like one in PFGE, although they originated from meat grown in different geographic areas of Sichuan province approximately 150 km apart. pIs were determined after comparison with the know pI of β-lactamase SHV12 (pI of 8.2, GenBank FJ685654). Both strains showed a single band with pI of approximately 8.2. This value corresponds to the OKP-A family, consistent with the OKP positive PCR result (Fevre *et al.,* 2005).

In our study, the prevalence of OKP among enterobacteria was low: the bla_{OKP} gene was detected in 4.10% (n=22) of all the isolates, among which two were from *S. marcescens* and 20 from *K. pneumonia.* OKP-producing *Serratia* species are rare, and this is the first report of two OKP-producing *S. marcescens* strains isolated from meat. The phenotypes of strains producing OKP enzymes were similar to those previously described for these types of β-lactamases (Fevre *et al.,* 2005; Hæggman *et al.,* 2004). The results of the present study clearly showed that OKP, previously found only in *K. pneumoniae*, has been detected in two *S. marcescens* related isolates from separate geographic locations. The study indicates that OKP enzymes are not *K. pneumoniae*-specific chromosomal β-lactamases and the *bla*OKP gene may be disseminated between different species.

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