Plasmid-borne 16S rRNA methylase ArmA in aminoglycoside-resistant *Klebsiella pneumoniae* in Poland

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We characterized 17 clinical isolates of *Klebsiella pneumoniae* producing 16S rRNA methylase ArmA. The isolates originated in Poland from 2002 to May 2010 and encompassed four *Xba*I-PFGE clusters. All the isolates were resistant to amikacin, gentamicin and kanamycin (MIC range: 256–1024 mg l⁻¹) and carried the *armA* gene on a large plasmid of approximately 90 or 130 kb in 15 and 2 isolates, respectively. The *armA* gene was found in a ~10 kb *Cla*I restriction fragment of the large plasmid and was flanked by the same elements as in Tn1548. All the isolates carried the *bla*_{CTX-M} gene for a CTX-M-type extended-spectrum β -lactamase. Our results show that ArmA has disseminated horizontally among *K. pneumoniae* isolates in Poland on the ~90 kb plasmid of the pCTX-M3 family.

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INTRODUCTION

Acquisition of multidrug resistance (MDR) by members of the *Enterobacteriaceae*, especially panaminoglycoside-resistant *Klebsiella pneumoniae*, has become a global concern. Aminoglycosides are commonly used to treat severe bacterial infections, often in combination with β -lactams (Mingeot-Leclercq *et al.*, 1999). Initially, production of aminoglycoside-modifying enzymes and a decrease in intracellular antibiotic accumulation were considered predominant mechanisms conferring resistance to aminoglycosides. The substitution of ribosomal proteins or mutation of rRNA were less commonly reported. In recent years, the emergence of panaminoglycoside-resistant bacteria producing plasmid-borne 16S rRNA methylases has been increasingly reported (Fritsche *et al.*, 2008). The methylases confer resistance to 4,6-disubstituted deoxystreptamines. This group encompasses the majority of aminoglycosides used in therapy (kanamycin, amikacin, gentamicin, netilmicin, tobramycin). Up to now, six 16S rRNA methylases have been described: ArmA, RmtA, RmtB, RmtC, RmtD and NpmA (Doi *et al.*, 2008). It is noteworthy that NpmA confers resistance not only to 4,6-disubstituted deoxystreptamines but also to 4,5-disubstituted 2-deoxystreptamines, including neomycin and ribostamycin (Wachino *et al.*, 2007).

ArmA (aminoglycoside resistance methyltransferase) was the first 16S rRNA methylase reported in a clinical isolate of *K. pneumoniae* (Galimand *et al.*, 2003). It was found in France in 2003. The *armA* gene encoding the methylase was detected in a composite transposon, Tn1548, located in a conjugative plasmid of approximately 90 kb (Galimand *et al.*, 2005). Nowadays, ArmA predominates in 16S rRNA methylase-producing members of the *Enterobacteriaceae* in Europe and is reported worldwide (Fritsche *et al.*, 2008). Although, the highest incidence (1.8%) of ArmA-producing strains in Europe has been reported in Bulgaria (Sabtcheva *et al.*, 2008), the incidence continues to increase in the world. Recently, 3% of clinical isolates of *K. pneumoniae* in China were reported to carry the *armA* gene (Wu *et al.*, 2009). The aim of this study was to determine the diversity of ArmA-producing *K. pneumoniae* in Poland. In addition, we identified a plasmid vector conferring ArmA production, and investigated the genetic environment surrounding the *armA* gene.

METHODS

Bacterial isolates. We conducted a retrospective screening of the 16S rRNA methylase-producing isolates in our stock of *K. pneumoniae* from hospitals and sanitary-epidemiological stations in Poland. This stock encompassed nearly 500 isolates of *K. pneumoniae* from a number of laboratories in the country. No epidemiological link was known for the majority of the isolates. Records of resistance

Abbreviations: ESBL, extended-spectrum β -lactamase; RFLP, restriction fragment length polymorphism.

profiles to aminoglycosides for nearly 300 isolates collected during the last 10 years (from April 2000 to May 2010) were screened in this study. In total, 34 isolates resistant to at least two aminoglycosides of the 4,6-disubstituted deoxystreptamines were found. Isolates resistant to a single agent were ignored. All the 34 isolates of *K. pneumoniae* were recovered from the stock and were reidentified by classical biochemical tube tests. ArmA-producing *Escherichia coli* with plasmid pMUR050 (González-Zorn *et al.*, 2005) and *E. coli* strain Δ 32/01 with *armA* in a large (approx. 90 kb) conjugative plasmid of the pCTX-M3 family were used as positive controls for 16S rRNA methylase production (Gierczyński *et al.*, 2003; Zacharczuk *et al.*, 2011).

Screening for 16S rRNA methylase-producing isolates. All 34 isolates were spread onto Mueller–Hinton agar supplemented with amikacin and kanamycin each at a concentration of 64 $\mu\text{g ml}^{-1}$.

Detection of resistance genes and the genetic environment of *armA*. Genes *armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD* and *npmA* encoding major 16S rRNA methylases were detected by PCR using primers and conditions described previously (Fritsche *et al.*, 2008). For the 16S rRNA methylase-producing isolates, amplification of extended-spectrum β -lactamase (ESBL)-encoding genes was performed with previously described primers (Dzierżanowska *et al.*, 2010) for *bla*_{TEM}, *bla*_{SHV} and *bla*_{CTX-M}. PCR mapping was conducted as described by Zhang *et al.* (2008). IncF and IncR primers described by Galimand *et al.* (2005) were used to detect plasmids of the IncL/M incompatibility group. DNA sequencing of PCR products was conducted for both the DNA strands as previously described (Gierczyński *et al.*, 2004). All the PCR tests were conducted in triplicate.

Susceptibility testing of 16S rRNA methylase-producing isolates. The MICs of gentamicin, amikacin, kanamycin, neomycin and streptomycin were determined for the 16S rRNA methylase-producing isolates by a serial double-dilution method in accordance with published recommendations (CLSI, 2008). *E. coli* with pMUR050 and *Salmonella enterica* serovar Oranienburg 32/01 were used as aminoglycoside-resistant controls. *E. coli* ATCC 25922 was used as a susceptible control for susceptibility testing.

Plasmid extraction and Southern blot hybridization analysis. A NucleoBond PC20 kit (Machery-Nagel) was used to extract plasmids from isolates producing 16S rRNA methylase. To investigate the *armA* gene localization, the purified total plasmids were separated on a 1.0% agarose gel (Prona Plus), then subjected to Southern blotting and hybridization with a probe specific for the *armA* gene. The purified total plasmids were also digested using the *Cla*I endonuclease equivalent *Bsu*151 (Fermentas Life Science) as recommended by the manufacturer. Restriction fragments were electrophoresed on a 1.5% agarose gel (Prona Plus). The resulting restriction fragment length polymorphism (RFLP) patterns were analysed by Southern blotting with the *armA* probe.

For Southern blot hybridization, plasmids or the RFLP patterns were transferred from electrophoretic gels to Nylonbind B membrane (SERVA Electrophoresis) and then hybridized with the *armA* gene specific probe. Probe labelling, hybridization and detection were carried out with a DIG High Prime DNA Labelling and Detection starter kit I (Roche Diagnostics) according to the manufacturer's instructions. Southern blot hybridization experiments were performed twice.

PFGE genotyping. To determine the genetic relatedness of the ArmA-producing *K. pneumoniae* isolates in Poland, we carried out PFGE. PFGE was conducted as described previously (Dzierżanowska *et al.*, 2010) using the CHEF-DR II system (Bio-Rad Laboratories) and endonuclease *Xba*I (Fermentas Life Science) with a switching time of 3–30 s for 24 h at 14 °C and voltage gradient of 6.0 V cm^{-1} .

PFGE patterns were analysed using the GelCompar II version 5.10 software (Applied Maths). Similarity clustering analyses were performed using UPGMA and the Dice correlation coefficient with a tolerance of 1.2%. PFGE genotyping was performed twice.

RESULTS

Potential 16S rRNA methylase producers

Among the 34 *K. pneumoniae* isolates screened, 17 grew on the selective Mueller–Hinton agar with amikacin and kanamycin. This result suggested potential 16S rRNA methylase activity in these 17 isolates. All these isolates were characterized in Table 1.

Aminoglycoside resistance profiles

The 17 isolates resistant to amikacin and kanamycin had elevated MICs (see Table 1) to all of the 4,6-disubstituted deoxystreptamines used in the study. The MIC range was high (256–1024 mg l^{-1}) and was comparable to MIC ranges reported elsewhere for 16S rRNA methylase-producing *K. pneumoniae* (Bogaerts *et al.*, 2007).

Screening of 16S rRNA methylase genes

All the 17 isolates resistant to amikacin and kanamycin were positive for the *armA* gene by PCR testing. No PCR products for *rmtA*, *rmtB*, *rmtC*, *rmtD* and *npmA* genes were detected in the tested isolates. DNA-sequencing analysis of the PCR products of two randomly selected isolates revealed 100% nucleotide identity to the *armA* gene of *K. pneumoniae* strain BM4536 (GenBank accession no. AY220558).

Screening of *bla* genes for the major β -lactamases

All the *armA*-positive isolates also carried the *bla*_{CTX-M} gene for an ESBL of the CTX-M-type family (Table 1). DNA sequencing of the *bla*_{CTX-M} gene using primers described previously (Gierczyński *et al.*, 2003) revealed that isolates 474/02 and DM0342 produced a CTX-M-3 ESBL. These isolates also carried the *bla* gene for a TEM-type β -lactamase. Eleven of the *armA*-positive isolates were also positive in the PCR with primers for the *bla*_{SHV} gene for an SHV-type enzyme (Table 1). The PCR product for IncL/M plasmids was detected in all ArmA-producing isolates of *K. pneumoniae* apart from 31/03 (Table 1).

Exploration of the regions surrounding the *armA* gene

PCR mapping experiments revealed that the *armA* gene and its flanking regions in all 17 *K. pneumoniae* isolates producing ArmA were in the same order and direction as in transposon Tn1548 found in *K. pneumoniae* in France

Table 1. Characterization of 17 *K. pneumoniae* isolates producing 16S rRNA methylase ArmA

Isolate	Year of isolation	Origin		MIC (mg l ⁻¹) of*					PCR†				Plasmid profile	PFGE cluster
		City	Hospital	GEN	AMK	KAN	STR	NEO	CTX-M	TEM	SHV	IncL/M		
468/02	2002	Łódź	H4	>1024	>1024	>1024	2	16	+	+	+	+	A	A
473/02	2002	Łódź	H1	>1024	>1024	>1024	2	16	+	+	+	+	A	B
474/02	2002	Łódź	H1	>1024	>1024	>1024	4	32	+	+	+	+	A	B
475/02	2002	Łódź	H1	>1024	>1024	>1024	2	16	+	+	+	+	B	B
477/02	2002	Łódź	H1	>1024	>1024	>1024	4	32	+	+	+	+	A	B
478/02	2002	Łódź	H1	>1024	>1024	>1024	2	16	+	+	+	+	B	B
2/03	2003	ND	ND	512	>1024	>1024	2	16	+	+	+	+	C	NA
12/03	2003	Łódź	H3	>1024	>1024	>1024	2	16	+	+	+	+	D	A
14/03	2003	Łódź	H3	>1024	>1024	>1024	2	8	+	+	+	+	B	A
30/03	2003	Łódź	H2	>1024	>1024	>1024	4	128	+	+	+	+	E	C
31/03	2003	Łódź	H2	256	>1024	>1024	2	32	+	+	-	-	E	C
177/03	2003	ND	ND	>1024	>1024	>1024	2	16	+	+	+	+	F	NA
DM0265	2009	Warsaw	H1	>1024	>1024	>1024	16	256	+	+	-	+	G	D
DM0266	2009	Warsaw	H1	>1024	>1024	>1024	16	256	+	+	-	+	H	D
DM0269	2009	Warsaw	H1	>1024	>1024	>1024	16	>512	+	+	-	+	G	D
DM0270	2009	Warsaw	H1	>1024	>1024	>1024	16	256	+	+	-	+	I	D
DM0342	2010	Warsaw	H2	>1024	>1024	>1024	32	2	+	+	-	+	A	NA

NA, Not assigned; ND, no data.*GEN, Gentamicin; AMK, amikacin; KAN, kanamycin; STR, streptomycin; NEO, neomycin.

†Results of PCR test for the *bla* genes encoding β -lactamases of the CTX-M, TEM and SHV families, and the IncL/M incompatibility group plasmids: +, positive result; -, negative result.

(GenBank accession no. AY220558) and *Citrobacter freundii* in Poland (GenBank accession no. AF550415).

Plasmid profiles, RFLP patterns and Southern blots

Fifteen isolates with the *armA* gene harboured two to six plasmids ranging from 3 to approximately 140 kb. Each of these isolates carried a ~90 kb plasmid. Two other isolates with the *armA* gene (30/03 and 31/03) possessed a large single plasmid of ~130 kb (Fig. 1a). For all 17 isolates, the *armA* probe hybridized exclusively to the large plasmid of ~90 or 130 kb. A background hybridization signal was observed for a band of approximately 23 kb corresponding to fragmented chromosome. Plasmid profiles and the corresponding Southern blots with the *armA* probe for three genetically unrelated isolates, 474/02, 2/03 and DM0265, are shown in Fig. 1(b). The *Cla*I (*Bsu*151) RFLP patterns of the plasmids extracted from each of the three aforementioned isolates are shown in Fig. 1(c). For the three unrelated isolates, the *armA* probe hybridized exclusively with a restriction fragment of approximately 10 kb in size.

PFGE genotyping

*Xba*I-PFGE patterns and a dendrogram of the pattern similarity for all 17 isolates of ArmA-producing *K. pneumoniae* are shown in Fig. 2. Fifteen isolates represented unique PFGE patterns. Two isolates were indistinguishable. Four clonal groups (A, B, C and D) encompassing 14 isolates

were distinguished. PFGE profiles of three isolates were distinct and could not be assigned to any of the clusters. Isolates of clusters B, C and D were hospital-specific, while cluster A isolates were specific for the city of Łódź.

DISCUSSION

K. pneumoniae is one of the major nosocomial pathogens. These bacteria effectively colonize patients as well as hospital personnel and occupy a hospital environment. To survive in this niche, *K. pneumoniae* developed a large variety of resistance traits including production of 16S rRNA methylases with the major representative ArmA (Fritsche *et al.*, 2008). In Europe, this methylase has been reported in *K. pneumoniae* from patients in France (Galimand *et al.*, 2003), Belgium (Bogaerts *et al.*, 2007) and Bulgaria (Sabtcheva *et al.*, 2008). In this study, we characterized 17 isolates of ArmA-producing *K. pneumoniae* in Poland. The isolates were collected from patients in six hospitals in the two most populated cities in the country: Warsaw and Łódź. To the best of our knowledge, this is the first report in which 16S rRNA methylase-producing *K. pneumoniae* isolates from various hospitals in Poland have been characterized. However, the ArmA-producing isolates from Warsaw, which we report herein, were also investigated in our recent study on factors conferring co-production of *K. pneumoniae* carbapenemase (KPC-2) and ArmA (Zacharczuk *et al.*, 2011).

In the present study, PFGE genotyping distinguished 16 genotypes among 17 ArmA-producing isolates of *K.*

Fig. 1. Electrophoretic patterns and Southern blots with the *armA* gene specific probe. (a) Plasmid profiles of 17 *K. pneumoniae* isolates producing ArmA. Designations of the profiles are shown in capital letters above the patterns. Lane J, profile of *E. coli* $\Delta 32/01$ with pCTX-M plasmid ~90 kb. (b) Plasmid profiles of three epidemiologically unrelated *K. pneumoniae* isolates and corresponding Southern blots. (c) *Bsu15I* (*Cln*) RFLP patterns of the plasmids shown in (b) together with the corresponding Southern blots. Lanes: 1, 2 and 3, *K. pneumoniae* isolates 474/02, 2/03 and DM0265M, respectively; M, linear dsDNA ladder – *HindIII* digest of the lambda phage (Sigma-Aldrich); L, DNA ladder – GeneRuler 1 kb Ladder (Fermentas Life Sciences). DNA size is shown in kb.

Fig. 2. Dendrogram of ArmA-producing *K. pneumoniae* isolates in Poland. The dendrogram is based on *XbaI*-PFGE pattern similarity.

pneumoniae collected during the past decade in Poland. The majority of these isolates were grouped to four PFGE clusters. In general, the PFGE clusters were hospital-specific. With a single exception, isolates of the same cluster were collected in the same hospital, and were closely related (similarity above 92%). These findings may reflect the nosocomial nature of *K. pneumoniae* infections and argue for an effective horizontal transfer of ArmA.

The hybridization experiments revealed that the *armA* gene was carried on a large ~90 kb plasmid in the majority of the tested *K. pneumoniae* isolates. However, in two related isolates, *armA* was detected on a larger plasmid of ~130 kb. All but one of the ArmA-producing *K. pneumoniae* isolates carried the IncL/M incompatibility group plasmid. The IncL/M plasmids encoding ArmA have been commonly reported for *K. pneumoniae* in France and in Bulgaria (Galimand *et al.*, 2005). Of note, in all the ArmA-producing *K. pneumoniae* isolates, the *armA* gene was found to be flanked by the same elements as reported previously in transposon Tn1548 (Galimand *et al.*, 2005). Since no difference in the elements adjacent to the *armA* gene was found between the 90 and 130 kb plasmid, this may suggest that *armA* has disseminated among *K. pneumoniae* isolates in Poland on the same transposable structure. Further studies are, however, necessary to elucidate the *armA* gene environment on these two large plasmids.

To characterize the 90 kb plasmid predominating among ArmA-producing *K. pneumoniae* in Poland, two isolates representative of two main *Xba*I-PFGE clusters (B and D), together with a third unrelated isolate, were selected for further RFLP and Southern blot experiments. Isolates of cluster B ($n=5$) were collected in 2002 in Łódź while isolates of cluster D ($n=4$) were collected in 2009 in Warsaw. The RFLP and Southern blot analysis revealed that the *armA* gene maps to the same approximately 10 kb *Cla*I (*Bsu*15I) restriction fragment of the large plasmid in all the three epidemiologically unrelated *K. pneumoniae* isolates. This finding, together with the results of the transposon mapping, may argue for a relatively conservative structure of the *armA* gene environment in *K. pneumoniae* in Poland. It is noteworthy that the presence of the *armA* gene in Tn1548 on a ~90 kb plasmid (pCTX-M3) was previously reported in *C. freundii* in Poland (Gołębiewski *et al.*, 2007) and in *K. pneumoniae* in France (Galimand *et al.*, 2005).

In a recent study, Zacharczuk *et al.* (2011) transferred by electroporation the 90 kb plasmid from ArmA-producing *K. pneumoniae* DM0269 to *E. coli* DH5 α . Interestingly, a large plasmid from recipient strain ETarmA belonged to the IncL/M incompatibility group and its *Cla*I-RFLP pattern closely resembled that of a large conjugative plasmid from CTX-M-3-producing *E. coli* transconjugant Δ 32/01 that was originally found in strain 32/01 of *S. Oranienburg* isolated in Poland in 2001 (Gierczyński *et al.*, 2003). It is noteworthy that the large plasmids derived from *K. pneumoniae* DM0269 and *S. Oranienburg* 32/01 carried the *armA* gene on a ~10 kb *Cla*I restriction

fragment (Zacharczuk *et al.*, 2011). These findings strongly support the results of the present study, and may argue for a conservative nature of the large plasmid on which the *armA* gene is horizontally transferred amongst *K. pneumoniae* and other *Enterobacteriaceae* species in Poland.

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