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Increased prevalence of aminoglycoside resistance in clinical isolates of *Escherichia coli* and *Klebsiella* spp. in Norway is associated with the acquisition of AAC(3)-II and AAC(6′)-Ib

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ABSTRACT

In this study, we show that the increasing prevalence of aminoglycoside resistance observed in Norway among clinical *Escherichia coli* and *Klebsiella* spp. isolates is mainly due to the presence of the aminoglycoside-modifying enzymes AAC(3)-II and AAC(6′)-Ib. A frequent co-association of aminoglycoside resistance with Cefotaximase-München group 1 extended-spectrum β-lactamases was also observed.

Aminoglycosides are an important group of antibiotics often used together with β-lactams in the treatment of severe infections caused by both Gram-negative and Gram-positive bacteria. Increasing aminoglycoside resistance among Gram-negatives is now threatening the clinical efficacy of these antibiotics. Aminoglycoside resistance in Gram-negatives is mainly conferred by production of aminoglycoside-modifying enzymes (AMEs) and 16S rRNA methylases (Ramirez and Tolmasky, 2010; Wachino and Arakawa, 2012). Genes encoding AMEs and 16S rRNA methylases are located on mobile genetic elements along with other resistance determinants such as extended-spectrum β-lactamases (ESBLs) and carbapenemases resulting in multi-drug resistant isolates (Ramirez and Tolmasky, 2010; Wachino and Arakawa, 2012). In Norway, gentamicin resistance has steadily increased during the last decade and has now reached ~5% among *Escherichia coli* and ~4% among *Klebsiella* spp. blood culture isolates in 2011, threatening the current national standard empirical treatment regimen for septicemia that includes gentamicin and a β-lactam (NORM/NORM-VET, 2012). Further, the prevalence of ESBLs among Gram-negative bacteria has also increased to similar levels (NORM/NORM-VET 2012). In this study, we have performed a molecular characterization of 2 nationwide strain collections to examine the molecular basis of aminoglycoside-resistant invasive *E. coli* and *Klebsiella* spp. and the potential association to the increasing prevalence of Cefotaximase-München (CTX-M)-type ESBLs.

Two strain collections were retrieved through the Norwegian surveillance program for antimicrobial resistance (NORM) from diagnostic microbiology laboratories (Table 1): i) the NORM-AMG collection consisting of all clinical isolates of *E. coli* (n = 105), *Klebsiella pneumoniae* (n = 31), and *Klebsiella oxytoca* (n = 1) from blood and urine samples, reported as resistant or intermediate susceptible to gentamicin and/or tobramycin among *E. coli* (n = 2510) and *Klebsiella* spp. (n = 1578) isolates included in the national surveillance program in 2009 (NORM/NORM-VET, 2010) and ii) the NORM-ESBL collection consisting of all ESBL-positive *E. coli* (n = 60) and *K. pneumoniae* (n = 8) clinical isolates from blood and urine in 2007–2008 (NORM/NORM-VET, 2008; NORM/NORM-VET, 2009). Species identification was performed using VITEK2 (bioMérieux, Marcy l'Etoile, France). All isolates were subjected to antimicrobial susceptibility testing using gentamicin, tobramycin, and amikacin E-tests according to the manufacturer's instructions (bioMérieux). The results were interpreted according to the clinical breakpoints of the European Committee on Antimicrobial Susceptibility Testing (www.eucast.org). Screening for genes encoding AMEs (*aac(6′)-Ib*, *aac(3)-IIa/c*, *aac(3)-Ia*, *ant(2″)-Ia*, and *ant(4′)-IIb*) and 16S rRNA methylases (*armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD*, *rmtE*, and *npmA*) was performed by PCR on isolates with reduced susceptibility to aminoglycosides (Table 2). The presence of CTX-M-ESBL genes was examined as previously described (Tofteland et al., 2007), and PCR products were

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Table 1
Summary of strain collections: relevant characteristics, prevalence of reduced susceptibility to aminoglycosides and distribution of AMEs.

Strain collection	Year	Species	Specimen	Total no. of isolates in NORM[a]	No. of isolates selected for molecular analysis[b]	No. of isolates (%) with reduced susceptibility[c]			No. of isolates positive for AME genes		
						GEN	TOB	AMK	*aac(6′)-Ib*	*aac(3)-II*	*ant(2″)-Ia*
NORM-AMG	2009	*E. coli*	Urine	1129	44	28 (2.5)	29 (2.6)	1 (0.1)	16	43	3
	2009	*E. coli*	Blood	1381	61	52 (3.8)	57 (4.1)	8 (0.6)	5	27	0
Total *E. coli*				2510	105	80 (3.2)	86 (3.4)	9 (0.4)	21	70	3
	2009	*Klebsiella* spp.	Urine	1007	15	7 (0.7)	10 (1.0)	2 (0.2)	9	14	0
	2009	*Klebsiella* spp.	Blood	571	17	8 (1.4)	11 (2.0)	2 (0.4)	13	14	0
Total *Klebsiella* spp.				1578	32	15 (1.0)	21 (1.3)	4 (0.3)	22	28	0
NORM-ESBL	2007–08	*E. coli*	Urine	3958	28	12 (43)	15 (54)	1 (4.0)	9	12	0
	2007–08	*E. coli*	Blood	2448	32	15 (47)	19 (59)	4 (13)	13	14	0
Total *E. coli*				6403	60	27 (45)	34 (56)	5 (8.3)	22	26	0
	2007–08	*Klebsiella* spp.	Blood	998	8	3 (38)	4 (50)	0 (0.0)	1	3	0

GEN = gentamicin; TOB = tobramycin; AMK = amikacin.
[a] Total number of isolates included in the NORM national surveillance program.
[b] The selection criteria for inclusion in the NORM-AMG collection included resistance or intermediate susceptibility to gentamicin and/or tobramycin. For the NORM-ESBL collection, the selection criteria included an ESBL phenotype and molecularly determined presence of a bla_{ESBL} gene.
[c] For the NORM-AMG collection, the total number of isolates in the NORM national surveillance program is used as the denominator for the calculations of prevalence of aminoglycoside reduced susceptibility. For the NORM-ESBL collection, the total number of ESBL-positive isolates is used as the denominator for the calculations of prevalence of aminoglycoside reduced susceptibility.

sequenced to determine the CTX-M group. *In vitro* conjugation experiments were performed as previously described (Samuelsen et al., 2009) using rifampicin resistant *E. coli* J53-2 as recipient. Transconjugants were selected on Luria-Bertani agar supplemented with i) 100 mg/L rifampicin, 6 mg/L gentamicin, and 2 mg/L cefotaxime; ii) 100 mg/L rifampicin + 2 mg/L cefotaxime; or iii) 100 mg/L rifampicin + 100 mg/L ampicillin. Transconjugants were subsequently analyzed by PCR for the presence of CTX-M and AME genes.

Antimicrobial susceptibility testing of the isolates with reported resistance or intermediate susceptibility to gentamicin and/or tobramycin in the NORM-AMG collection revealed that 18% and 31% of the *E. coli* and *Klebsiella* spp. isolates, respectively, were susceptible to the aminoglycosides tested. These results confirm a prevalence of reduced susceptibility to gentamicin, tobramycin, and amikacin in *E. coli* to be 3.2%, 3.4%, and 0.4%, respectively, among all isolates (n = 2510) included in the national surveillance program (Table 1). For *Klebsiella* spp., the prevalence of reduced susceptibility was 1.0%, 1.3%, and 0.3% to gentamicin, tobramycin, and amikacin, respectively, among all isolates (n = 1578). In both *E. coli* and *Klebsiella* spp., the prevalence of reduced susceptibility was higher in blood culture isolates than in urine isolates (Table 1), but this was only statistically significant for tobramycin and *E. coli* ($P = 0.04$ by Fisher's exact test). Co-resistance to gentamicin and tobramycin was commonly observed with 93% of *E. coli* and 64% of *Klebsiella* spp. isolates non-susceptible to aminoglycosides showing reduced susceptibility to both gentamicin and tobramycin. A total of 20% and 31% of the *E. coli* and *Klebsiella* spp., isolates resistant or intermediate susceptible to gentamicin and/or tobramycin, respectively, in the NORM-AMG collection produced an ESBL.

In the NORM-ESBL collection, the prevalence of reduced susceptibility to aminoglycosides was significantly higher ($P < 0.001$ by Fisher's exact test) than in the overall NORM 2009 collection. A total of 45%, 57%, and 8.3% of the *E. coli* isolates displayed reduced susceptibility to gentamicin, tobramycin, and amikacin, respectively (Table 1). No *Klebsiella* spp. isolates showed reduced susceptibility to amikacin, whereas for gentamicin and tobramycin the results were similar to *E. coli* with 38% and 50% non-

Table 2
Primers used in the study.

Name	DNA sequence 5′-3′	Target site	Amplicon size (bp)	Reference
aac(6′)-Ib-F	TTGCGATGCTCTATGAGTGGCTA	*aac(6′)-Ib*	482	(Park et al., 2006)
aac(6′)-Ib-R	CTCGAATGCCTGGCGTGTTT			
aac(3)-Ia_F	ATGGGCATCATTCGCACATGTAGG	*aac(3)-Ia*	465	(Hujer et al., 2006)
aac(3)-Ia_R	TTAGGTGGCGGTACTTGGGTC			
aac(3)-II-F	TGAAACGCTGACGGAGCCTC	*aac(3)-IIa/c*	370	(Jensen et al., 2006)
aac(3)-II-B	GTCGAACAGGTAGCACTGAG			
ant(2″)-Ia_F	ATGGACACAACGCAGGTCGC	*ant(2″)-Ia*	535	(Hujer et al., 2006)
ant(2″)-Ia_R	TTAGGCCGCATATCGCGACC			
ant(4′)-IIb_F	TATCTCGGCGGCGGTCGAGT	*ant(4′)-IIb*	364	This study
ant(4′)-IIb_R	CACGCGGGGAAACGCGAGAA			
rmtB-F	GCTTTCTGCGGGCGATGTAA	*rmtB*	173	(Doi and Arakawa, 2007)
rmtB-R	ATGCAATGCCGCGCTCGTAT			
rmtC-F	CGAAGAAGTAACAGCCAAAG	*rmtC*	711	(Doi and Arakawa, 2007)
rmtC-R	ATCCCAACATCTCTCCCACT			
armA-F	ATTCTGCCTATCCTAATTGG	*armA*	315	(Doi and Arakawa, 2007)
armA-R	ACCTATACTTTATCGTCGTC			
rmtA-F	CTAGCGTCCATCCTTTCCTC	*rmtA*	635	(Doi and Arakawa, 2007)
rmtA-R	TTGCTTCCATGCCCTTGCC			
rmtD-F	CGGCACGCGATTGGGAAGC	*rmtD*	401	(Doi and Arakawa, 2007)
rmtD-R	CGGAAACGATGCGACGAT			
rmtE-F	ATGAATATTGATGAAATGGTTGC	*rmtE*	818	(Davis et al., 2010)
rmtE-R	TGATTGATTTCCTCCGTTTTTG			
npmA-F	CTCAAAGGAACAAAGACGG	*npmA*	640	(Doi and Arakawa 2007)
npmA-R	GAAACATGGCCAGAAACTC			
ctx-m-F	SCSATGTGCAGYACCAGTAA	bla_{CTX-M}	585	(Tofteland et al., 2007)
ctx-m-R	ACCAGAAYVAGCGGBGC			