Genetic identity of aminoglycoside-resistance genes in *Escherichia coli* isolates from human and animal sources

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A bacterial collection ($n=249$) obtained in Hong Kong from 2002 to 2004 was used to investigate the molecular epidemiology of aminoglycoside resistance among *Escherichia coli* isolates from humans and food-producing animals. Of these, 89 isolates were gentamicin-sensitive (human $n=60$, animal $n=29$) and 160 isolates were gentamicin-resistant (human $n=107$, animal $n=53$). Overall, 84.1 % (90/107) and 75.5 % (40/53) of the gentamicin-resistant isolates from human and animal sources, respectively, were found to possess the *aacC2* gene. The *aacC2* gene for 20 isolates (10 each for human and animal isolates) was sequenced. Two alleles were found that were equally distributed in human and animal isolates. PFGE showed that the gentamicin-resistant isolates exhibited diverse patterns with little clonality. In some isolates, the *aacC2* gene was encoded on large transferable plasmids of multiple incompatibility groups (IncF, IncI1 and IncN). An IncFII plasmid of 140 kb in size was shared by one human and three animal isolates. In summary, this study showed that human and animal isolates share the same pool of resistance genes.

Received 4 August 2009
Accepted 22 February 2010

INTRODUCTION

Urinary tract infection is one of the most frequent diagnoses in the outpatient setting. It has been estimated to affect 40–50 % of women in their lifetime and recurrence is common. *Escherichia coli* is by far the most common uropathogen, accounting for 75–95 % of all positive cultures in uncomplicated cystitis (Ho *et al.*, 2009). Several recent studies suggest that resistance to ampicillin (Canica *et al.*, 2004; Manges *et al.*, 2007), co-trimoxazole (Ramchandani *et al.*, 2005) and the fluoroquinolones (Johnson *et al.*, 2006) in some uropathogenic *E. coli* may have originated from food-producing animals, possibly involving the horizontal transfer of genes conferring virulence and antibiotic resistance (Johnson & Nolan, 2009; Skyberg *et al.*, 2006). Furthermore, it has been suggested that bacterial isolates from food-producing animals could be an important reservoir for the aminoglycoside-resistance genes found in human isolates (Chaslus-Dancla *et al.*, 1991; Johnson *et al.*, 1994, 1995).

Despite the existence of more than 85 aminoglycoside-modifying enzymes, only a limited number of them, ANT(2'')-I, AAC(6')-I, AAC(3)-I, AAC(3)-II, AAC(3)-III, AAC(3)-IV and AAC(3)-VI, appear to have undergone selection and cause the majority of aminoglycoside resistance (Miller *et al.*, 1997; Vakulenko & Mobashery,

2003). In *E. coli*, gentamicin resistance is most commonly mediated by AAC(3)-I, AAC(3)-II, AAC(3)-IV and ANT(2'')-I enzymes (Miller *et al.*, 1997; Vakulenko & Mobashery, 2003). Although there are many ongoing surveillances of antimicrobial resistance among *E. coli* isolates in animals, little attempt has been made to correlate the aminoglycoside-resistance mechanisms with those in human-associated, clinical isolates.

In Hong Kong, recent antimicrobial-resistance surveillance showed that the prevalence of gentamicin resistance in human-associated, urinary *E. coli* and animal-associated, faecal *E. coli* were 21 and 21.8–57.2 %, respectively (Duan *et al.*, 2006; Ho *et al.*, 2007b). Susceptibility testing showed that most isolates from human and animals exhibited the GTN resistance phenotype (resistant to gentamicin, tobramycin and netilmicin; sensitive to amikacin). Hence, this study was conducted to investigate the relationship between aminoglycoside resistance among *E. coli* isolates obtained from human-associated, urinary isolates and food-producing animal-associated, faecal isolates.

METHODS

Bacterial strains. A total of 249 isolates including 89 gentamicin-sensitive and 160 gentamicin-resistant *E. coli* isolates obtained from

humans and food-producing animals were studied. The isolates were obtained from previous surveillance studies of antimicrobial resistance (Duan *et al.*, 2006; Ho *et al.*, 2007a, 2008). A subset of 249 isolates including 103 urinary isolates from female outpatients with uncomplicated urinary tract infections, 82 faecal isolates from food-producing animals, and 64 faecal isolates from children and adults (Duan *et al.*, 2006; Ho *et al.*, 2007a, 2008). The isolates were chosen among 1239 *E. coli* strains according to the most common antimicrobial-resistance phenotypes for the following antibiotics: ampicillin, amoxicillin–clavulanate, gentamicin and trimethoprim–sulfamethoxazole. The food-producing animals from which the isolates were recovered were imported to Hong Kong from the southern part of mainland China. All the human and food-producing animal isolates were obtained from samples obtained in Hong Kong during 2002–2004. The proportion of gentamicin resistance among the different categories of isolates in the original collection and the number of isolates chosen for analysis are summarized in Table 1.

Bacterial identification and antimicrobial-susceptibility testing.

Bacteria were identified by biochemical tests and the VITEK GNI system (bioMérieux). Antimicrobial susceptibility testing was performed by the disc diffusion method in accordance with Clinical and Laboratory Standard Institute recommendations (CLSI, 2007). All antimicrobial discs were obtained commercially (BBL; Becton Dickinson).

Molecular analysis. PCR was conducted with primers described for the detection of genes encoding the four AAC(3) isoenzymes and ANT(2)-I (Vanhoof *et al.*, 1992; Vliegenthart *et al.*, 1990, 1991). In a preliminary analysis, it was shown that AAC(3)-II was the predominant enzyme present, while AAC(3)-I, AAC(3)-III, AAC(3)-IV and ANT(2)-I were either absent or found rarely. Therefore, the full scale analysis focused on detection of AAC(3)-II. For nucleotide sequencing of the *aacC2* gene, the following primer pair was designed in this study: forward, 5'-TAG AGG AGA TAT CGC GAT GC-3' (position 75–94 GenBank accession no. X51534); and backward,

5'-ATT ATC ATT GTC GAC GGC CT-3' (position 971–952 GenBank accession no. X51534). Both strands of the amplicons were sequenced. Conjugation experiments were carried out on sterilized filters with *E. coli* J53Az^r as the recipient (Ho *et al.*, 2005b). Donor and recipient cells were mixed at a ratio of 1:10. Transconjugants were selected on Muller–Hinton agar plates containing gentamicin (10 µg ml⁻¹) together with sodium azide (150 µg ml⁻¹). The epidemiological relatedness of the donor isolates were studied by PFGE of *Xba*I-digested genomic DNA (Amersham Pharmacia Biotech) and patterns were analysed with GelCompar II software (Applied Maths) (Ho *et al.*, 2007a). Dendrograms were created by means of the Dice coefficient and the UPGMA method. Band position and optimization were set at 1.5 and 1%, respectively. A similarity coefficient of 85% was selected to define clonal clusters (Ho *et al.*, 2007a).

Small plasmids (less than 10–20 kb) were extracted with the High Pure plasmid isolation kit (Roche Diagnostics) and analysed by conventional gel electrophoresis. Large plasmids were sized by the S1 nuclease and PFGE techniques as previously described (Ho *et al.*, 2005a). The PCR-based replicon typing method described previously was used to determine the incompatibility (Inc) groups of plasmids carried by *E. coli* transconjugants (Carattoli *et al.*, 2005). The method allowed recognition of the following 18 incompatibility groups: FIA, FIB, FIC, HII, HI2, II-I₇, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, and FIIA. An additional primer pair was used for identification of IncFII replicons (Osborn *et al.*, 2000). Identification of the plasmid replicons was confirmed by sequencing of the PCR products.

RESULTS AND DISCUSSION

Prevalence of *aacC2* genes and PFGE typing of strains

A total of 249 isolates, including 160 gentamicin-resistant and 89 gentamicin-sensitive isolates were studied. Overall, 81.3% (130/160) of the gentamicin-resistant isolates were PCR positive for the *aacC2* gene. The proportions were 84.1% (90/107) and 75.5% (40/53) for human and animal isolates, respectively ($P=0.2$; Table 2). All 89 gentamicin-sensitive isolates were *aacC2* negative. The *aacC2* gene for 20 isolates (10 each for human and animal isolates) was amplified and sequenced. The result showed that there were two *aacC2* allelic variants, designated *aacC2d* and *aacC2e* (encoding AAC(3)-IId and AAC(3)-IIe, respectively). The predicted amino acid sequence of the two alleles differed from AAC(3)-IIa by approximately 5% (Fig. 1). Both *aacC2* variants were shared by human and animal isolates, and the representation in both groups was similar. A total of 48 of the 130 *aacC2* isolates was analysed by PFGE (Fig. 2). The result showed that the isolates were genetically diverse and only a small proportion of the isolates exhibited a clonal relationship. The human and animal isolates with *aacC2* genes were not clonally related.

The findings showed that gentamicin resistance is caused by the same mechanism among *E. coli* isolates in human and food-producing animals. There was widespread presence of the *aacC2* gene in isolates from human and food-producing-animal sources in Hong Kong. The PFGE analysis showed that there was only limited clonal spread. However, there was sharing of the AAC(3)-II alleles by *E.*

Table 1. Sources of the 249 isolates chosen for analysis and the proportion of gentamicin resistance among the original bacterial collections

Source	No. (%) of isolates chosen for analysis/no. in category		
	Gen-S	Gen-R	Both
Human			
Urine	30/468	73/147	103/615 (16.7)
Faecal	30/36	34/35	64/71 (90.1)*
Subtotal	60/504	107/182	167/686 (24.3)
Animal			
Poultry	12/79	25/122	37/201 (18.4)
Pig	12/206	21/69	33/275 (12)
Cattle	5/56	6/20	11/76 (14.5)
Fish	0/0	1/1	1/1
Subtotal	29/341	53/212	82/553 (14.8)
Total	89/845	160/394	249/1239 (20.1)

*Including 22 isolates from adults and 42 isolates from children. The total number of isolates available from adults and children was 27 and 44, respectively.

Table 2. Frequency of *aacC2* gene among gentamicin-resistant *E. coli* isolates, according to source of isolate

Source	No. <i>aacC2</i> -positive/no. tested (%)		Allelic variant found (no. of isolates)*
	Gen-S	Gen-R†	
Human			
Urine	0/30 (0)	62/73 (84.9)	<i>aacC2d</i> (4), <i>aacC2e</i> (3)
Faecal	0/30 (0)	28/34 (82.4)	<i>aacC2d</i> (2), <i>aacC2e</i> (1)
Subtotal	0/60 (0)	90/107 (84.1)	
Animal			
Poultry‡	0/12 (0)	18/25 (72)	<i>aacC2e</i> (4)
Pig	0/12 (0)	15/21 (71.4)	<i>aacC2d</i> (2), <i>aacC2e</i> (1)
Cattle	0/5 (0)	6/6 (100)	<i>aacC2d</i> (2)
Fish	0	1/1 (100)	<i>aacC2e</i> (1)
Subtotal	0/29 (0)	40/53 (75.5)	
Total	0/89 (0)	130/160 (81.3)	

Gen-S, Gentamicin-sensitive; Gen-R, gentamicin-resistant.
*The genes in 20 isolates were sequenced.
†No significant difference between positive rates in human and animal isolates ($P=0.2$, χ^2 test).
‡The 37 isolates included 19 isolates from chicken, 10 from geese, 4 from ducks and 4 from pigeons.

Fig. 1. Multiple sequence alignment of the amino acid sequences of AAC(3)-II isoenzymes. The GenBank accession numbers were as follows: AAC(3)-IIa, X13543; AAC(3)-IIb, M97172; AAC(3)-IIc, X54723; AAC(3)-IId, EU022314 (this study); and AAC(3)-Ile, EU022315 (this study). The deduced protein sequences were aligned using the CLUSTAL W program. The dots indicate identical amino acid residues compared with AAC(3)-IIa; the dashes indicate amino acids that are absent.

Fig. 2. Dendrogram showing the genetic relatedness of 48 *E. coli* isolates tested positive for the *aacC2* gene. The boxes indicate small clusters at $\geq 85\%$ similarity (UPGMA; Dice, black vertical line) (Ho *et al.*, 2007a). UTI, Urinary tract infection.

coli of human and animal origins. In Belgium, the gene encoding AAC(3)-IV was found among isolates from food-producing animals and humans (Pohl *et al.*, 1993). Our conjugation experiment showed that the pattern of acquired resistance to the aminoglycosides (GTN) was similar to that reported for the AAC(3)-II enzymes in general (Vakulenko & Mobashery, 2003). In agreement with other reports (Vakulenko & Mobashery, 2003; Vliegenthart *et al.*, 1989), the high frequency of *aacC2* in genetically diverse strains suggests that horizontal gene transfer plays a larger role than clonal expansion in the increase of gentamicin-resistance levels in this region.

The complete amino acid sequences of both AAC(3)-II variants were compared against GenBank sequences by BLAST search (GenBank accession no., source, year of GenBank submission). The sequence of AAC(3)-IId was 100 % identical to those found in: *Citrobacter freundii* from

Poland (AAN87703, human, 2002), *Enterobacter cloacae* from Spain (ACO57223, human, 2009), *Klebsiella pneumoniae* from Taiwan (ABG56862, human, 2006), *Salmonella typhimurium* from the USA (AAR05727, clinical isolate, 2003), *Serratia marcescens* from Korea (AAA19915, clinical isolate, 1992) and *Actinobacillus pleuropneumoniae* from China (ACD76079, pig, 2008). No sequence in the GenBank had 100 % identity with AAC(3)-IId. However, highly similar (98 % identity) sequences were found in clinical isolates of: *E. coli* from Spain (AAB20442), the UK (ACQ41898) and Canada (AAR25031), and in *K. pneumoniae* from Uruguay (ACF06169), *Enterobacter cloacae* from the Netherlands (CAA35913), *Acinetobacter baumannii* from South Africa (AAN34370) and *Pseudomonas aeruginosa* from France (AAN61405). The presence of AAC(3)-IId and AAC(3)-IId in a variety of bacterial species from different geographical regions supports their global distribution and that cross-species transfer via mobile genetic elements may occur frequently.

Conjugation and replicon typing of plasmids carrying *aacC2* genes

In the conjugation experiment, transferable gentamicin resistance was demonstrated in 14 of 40 strains tested (Table 3). The frequency of transfer ranged from 10^{-3} to 10^{-5} per donor cell. The transconjugants exhibited acquired resistance to gentamicin, tobramycin and netilmicin, but retained sensitivity to amikacin. To confirm the plasmid location of the *aacC2* gene, the plasmid DNA bands from the S1-PFGE were excised from the gel and used as DNA templates for PCR. The result revealed that *aacC2* genes were encoded in plasmids with sizes ranging from 80 to 270 kb. Replicon typing showed that plasmids carrying *aacC2* had diverse genetic backgrounds, including incompatibility groups with narrow (IncF and IncI1) and broad host ranges (IncN). An IncFII plasmid with

approximately 140 kb size was found to be shared by one human and three animal isolates.

Replicon typing of the transmissible plasmids indicated that the *aacC2* genes [AAC(3)-IId and AAC(3)-IId] are often encoded in IncF plasmids, as has been reported for the *aacC2* gene encoding AAC(3)-IId (Karisik *et al.*, 2006). Our findings showed that *aacC2* genes were also found among IncI1 and IncN plasmids. By comparison, other studies have demonstrated *aacC4* genes on Inc9 and IncQ (Chaslus-Dancla *et al.*, 1991). Given that *aacC2* may be located on transposons and integrons (Vakulenko & Mobashery, 2003), additional mechanisms may be involved in the horizontal transfer of the genes in different bacterial populations; thus providing a possible explanation for its detection in plasmids of multiple replicon types. Since only a small number of isolates from a highly selected sample were tested for the plasmid replicon types and were sequenced for the *aacC2* alleles, no firm conclusion could be drawn about their relative distributions. In future studies, it would be useful to determine if the *aacC2* genes were carried on plasmids with virulence factors that are commonly carried by the so-called avian pathogenic *E. coli* (Johnson & Nolan, 2009).

In summary, our findings showed that a substantial proportion of the gentamicin resistance in *E. coli* outpatient urinary isolates was attributed to resistance genes that are widespread among faecal isolates obtained from food-producing animals. The observation provides further support to concerns about transmission of resistance between food-producing animals and humans. This can occur via multiple routes of which direct transmission via consumption of meat and eggs is probably most important (Manges *et al.*, 2007; Musgrove *et al.*, 2006). Other potential modes of transmission include direct contact with live animals, their environment or exposure to contaminated water sources (Aarestrup, 2006).

Table 3. Replicon typing of conjugative plasmids carrying the *aacC2* gene from 14 *E. coli* isolates

Transconjugant	Source	Transferred plasmid (kb)*	Plasmid replicon typing
53T1G-RAN39	Human	<u>140</u>	FII, F1B
jO68 g	Cattle	<u>140</u>	FII, F1B
jG28-1 g	Goose	80, <u>140</u>	FII, F1B
jPI056-2 g	Pig	<u>140</u>	FIA, FIB
53T1G-chicken36	Chicken	<u>150</u>	FII, F1B
53T1G-chicken37	Chicken	<u>180</u>	FII, F1B
jPI046 g	Pig	<u>80</u>	FII
jPI067 g	Pig	<u>90</u>	FII
j37 g	Chicken	120, 160	II-I γ , N, FIB, FII
jG27 g	Goose	<u>110</u>	II-I γ
jG30-1 g	Goose	<u>110</u>	II-I γ
53T2G-chicken188	Chicken	<u>270</u>	N
53T1G-PigPI010	Pig	120, 175	N, FII
53T1G-F04-1	Fish	<u>270</u>	Undetected

*Plasmids carrying *aacC2* genes are underlined.

ACKNOWLEDGEMENTS

This work was supported by research grants from the Research Grant Council (HKU 7513/06M) and the University Development Fund Project – Research Centre of Emerging Infectious Diseases, the University of Hong Kong; and the Research Fund for the Control of Infectious Diseases (RFCID) of the Health, Welfare and Food Bureau of the Hong Kong SAR Government. We thank the University of Hong Kong for the Outstanding Research Postgraduate Student award to River C. Wong.

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