High frequency transfer and horizontal spread of apramycin resistance in calf faecal Escherichia coli

C. M. Yates*, M. C. Pearce, M. E. J. Woolhouse and S. G. B. Amyes

1Medical Microbiology, University of Edinburgh Medical School, Edinburgh EH8 9AG; 2Centre for Tropical Veterinary Medicine, University of Edinburgh, Roslin, Midlothian EH25 9RG, UK

Received 13 April 2004; returned 8 May 2004; revised 7 June 2004; accepted 7 June 2004

Objectives: The aminoglycoside apramycin has been used extensively in animal husbandry in the UK since 1978. This study aimed to determine both whether calves that had never been treated with aminoglycoside antibiotics harboured apramycin-resistant (aprR) commensal Escherichia coli, and the mode of spread of the resistance gene.

Methods: AprR E. coli from weekly calf faecal samples were typed by pulsed-field gel electrophoresis, antibiotic resistance phenotype, plasmid restriction profiles and plasmid transfer frequencies.

Results: During 4 months of weekly sampling, six of 11 calves were found to harbour aprR E. coli. All aprR E. coli (45) were cross-resistant to gentamicin and tobramycin, which are both used in human medicine. Resistance was conferred by the aac(3)IV gene, present on three different conjugative plasmids. Two of these plasmids also mediated tetracycline and streptomycin resistance. One plasmid demonstrated very high transfer frequencies and was found in three different genotypes.

Conclusions: We report the presence of aprR commensal E. coli in cattle that have never been treated with aminoglycosides. The presence of one conjugative plasmid in three different genotypes is evidence of horizontal spread of this plasmid. This is the first report of a very high transfer frequency of aprR plasmid, demonstrating horizontal spread in the commensal flora of food animals.

Keywords: conjugation, commensals, AAC(3)IV, food animals, plasmids

Introduction

The study of antibiotic-resistant bacteria in animals has focused on organisms pathogenic to humans and animals, such as salmonellae and campylobacter.1 Resistance genes commonly reside on transmissible plasmids, transposons, gene cassettes or other mobile genetic elements, allowing the horizontal spread of resistance genes between strains, species and even genera. Because of this genetic mobility, the commensal flora of animals may act as a reservoir of resistance genes.

Apramycin has been used extensively in animal husbandry since 1978. Although it has not been used in human medicine, apramycin resistance has been detected in human isolates of Klebsiella pneumoniae and Escherichia coli.2 These findings, in contrast to a recent report by Phillips et al.,1 indicate that the gene conferring resistance appears to have transferred between animal and human bacteria. Apramycin resistance is conferred by the aminoglycoside-modifying enzyme 3-N-aminoglycoside acetyltransferase type IV [AAC(3)IV],3 which also acetylates tobramycin and gentamicin, used to treat serious infections in humans.

This study aimed to determine whether calves that had not been treated with aminoglycosides harboured aprR commensal E. coli, and whether apramycin resistance was spread clonally or horizontally.

Materials and methods

Bacterial isolates

Rectal faecal samples from 11 beef-suckler calves on a Scottish farm, born between 14–26 September 2001, were taken within 48 h of birth, then weekly until 14 January 2002 (except for 2 weeks at Christmas and New Year). Enrofloxacin, florfenicol, oxytetracyline, penicillin, streptomycin and tylosin were used on the farm during the sampling period, but no tetracyclines or aminoglycosides were used on the sampled calves. Samples were stored at 4°C and processed within 24 h of collection. Samples were diluted 1:10 in maximum recovery diluent (Oxoid, Basingstoke, UK). Ten millilitres of

*Corresponding author. Tel: +44-131-650-8270; Fax: +44-131-651-1385; E-mail: cyates@ed.ac.uk

JAC vol.54 no.2 © The British Society for Antimicrobial Chemotherapy 2004; all rights reserved.
Apramycin resistance plasmids in commensal *E. coli*

Each suspension was spread onto Chromocult TBX (tryptone bile X-glucuronide) agar (Merck, Darmstadt, Germany), supplemented with apramycin 16 mg/L, and cultured overnight at 44°C. One to 10 aprR glucuronidase-positive (blue) *E. coli* were picked from each plate showing growth, purified by subculturing twice on MacConkey agar (Oxoid) supplemented with apramycin 16 mg/L, then stored at −70°C on cryogenic beads (Mast Diagnostics, Germany).

**Antibiotic susceptibility**

MICs of ampicillin, apramycin, gentamicin, streptomycin, sulfamethoxazole, tetracycline (all from Sigma, Poole, UK) and tobramycin (Faulding Pharmaceuticals, Warwickshire, UK) were determined following BSAC guidelines.4

**Pulsed-field gel electrophoresis (PFGE)**

Preparation of genomic DNA and digestion with XbaI was performed as described by Gautom.5 Samples were electrophoresed on 1% agarose gels using a CHEF DRII machine (Bio-Rad), with initial gradient, 6.0 V/cm; temperature, 14°C; ramping factor, linear. Gels were stained in distilled water containing 1 mg/mL ethidium bromide and photographed with the Gel-Doc 2000 system (Bio-Rad). Preparations of genomic DNA and digestion with XbaI were denatured for 5 min at 94°C, followed by 30 cycles of 1 min denaturation (94°C), 1 min anneal (55°C) and 1 min extension (72°C). Sequencing was performed by chain termination in both directions and compared with the published sequence.3

**Cluster analysis**

PFGE fingerprints were compared by the Dice coefficient, and clustered by the unweighted pair group method using arithmetic averages (UPGMA) in BioNumerics v.2.5 software (Applied Maths, Sint-Martens-Latem, Belgium). Cluster analysis was performed with a position tolerance and optimization of 1%.

**Conjugation experiments**

AprR plasmids were initially transferred into *E. coli* J62-2 by mating aprR isolates with J62-2 in a 1:100 ratio, respectively, overnight at 37°C in nutrient broth (NB) (Oxoid).6 Transfer frequencies, completed in triplicate, were defined as the proportion of transconjugants over total number of donors (D) at the start of mating,6 and were completed in triplicate, were defined as the proportion of transconjugants over total number of donors (D) at the start of mating,6 and were measured between J62-2(D) and the related auxotroph J53.6 Over- 

<table>
<thead>
<tr>
<th>Calf</th>
<th>Treatment (date)</th>
<th>Sampling dates of aprR E. coli detection</th>
<th>AprR E. coli isolates</th>
<th>PFGE types (number of each type)</th>
</tr>
</thead>
<tbody>
<tr>
<td>690</td>
<td>none</td>
<td>01/10/2001, 12/11/2001</td>
<td>2</td>
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<tr>
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<tr>
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<td>none</td>
<td>26/09/2001</td>
<td>8</td>
<td>C (8)</td>
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<tr>
<td>696</td>
<td>none</td>
<td>12/11/2001</td>
<td>6</td>
<td>A (1), B(4), D (1)</td>
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<td>enrofloxacine (01/10/2001)</td>
<td>10/12/2001</td>
<td>1</td>
<td>A (1)</td>
</tr>
</tbody>
</table>

**Results**

Although no aminoglycoside antibiotics were used on the 11 calves sampled, six calves harboured aprR *E. coli* (≥1000 cfu/mL). Detection dates, calf identities, antibiotic treatment records and genotypes of aprR *E. coli* are listed in Table 1. Forty-five aprR *E. coli* were isolated. Genotyping by PFGE revealed five distinct aprR genotypes (Table 1), differing from each other by up to 46% (results not shown). Variation within PFGE types was only found in type A: a 563 kb band was present in two (2/27) isolates compared with a 531 kb band in the remaining isolates. In the majority (7/9) of samples, only one genotype was detected (Table 1), but in samples from calves 693 and 696 two and three different genotypes, respectively, were found. Type A isolates were the most common, and carried tetracycline, sulfamethoxazole, apramycin, tobramycin and gentamicin resistance.

Plasmid profiling with EcoRI and ApaI revealed three conjugative plasmids: pUK2001, pUK2002 and pUK2003, of size 91, 115 and 181 kb, respectively (Table 2). Plasmid pUK2001 was found in three different genotypes, indicating horizontal spread.
We were unable to restrict pUK2003, but the different sizes and transfer frequencies (Table 2) indicate that pUK2003 and pUK2002 are different plasmids. pUK2001 transferred at the highest frequency (1.15 × 10^{-2} h^{-1}) (Table 2), which was substantially greater than that of RP4 and R46 (1.4 × 10^{-3} h^{-1} and 3.1 × 10^{-4} h^{-1}, respectively). MICs for the donor strains, recipient and transconjugants revealed that pUK2002 and pUK2003 also carried tetracycline and streptomycin resistance (Table 2). This was confirmed by the observation of similar transfer frequencies when pUK2002 or pUK2003 were selected on tetra-cyclines, streptomycin or each of these antibiotics in combination with apramycin (data not shown). In addition to apr R (MIC >128 mg/L) all three plasmids conferred resistance to tobramycin and gentamicin (MICs of 16 and 8 mg/L, respectively). All three plasmids harboured the aac(3)-IV gene. The sequences were identical to that of the published nucleotide sequence (X01385).

Discussion

Previous reports on apramycin resistance have concentrated on human or animal pathogens. Little is known about the epidemiology of resistant commensal bacteria. In this study, PFGE and plasmid analysis were combined to give a better understanding of the spread of apramycin resistance.

A cohort of calves that had not been treated with any aminoglycosides was selected for this work, to avoid any direct selection of apr R E. coli during the study. During 4 months, six of 11 calves were found to carry apr R E. coli.

The farm from which the isolates used in this study came had not used apramycin since July 2000 (when treatment records commenced). The carriage of streptomycin and tetracycline resistance by pUK2002 and pUK2003 suggests either cross-contamination of these antibiotics between calves, or the spread of resistance plasmids or strains between calves. The presence of apr R plasmids in the calves sampled in this study demonstrates the persistence of apramycin resistance without direct selective pressure by the use of apramycin. A similar result has been reported in E. coli from disease outbreaks in cattle, pigs, sheep and poultry. Two apr R E. coli isolates from diseased cattle were found to transfer apramycin resistance at frequencies of <10^{-8} and <10^{-10} transconjugants per donor overnight. In addition to the high transfer frequencies of pUK2001 and pUK2002 observed in this study, the use of PFGE to genotype each isolate allowed the detection of pUK2001 in three different genotypes. To the best of our knowledge, this is the first report of horizontal spread of apramycin resistance in commensal organisms.

The selective pressures maintaining these large conjugative resistance plasmids in the commensal flora are unclear. The calves sampled were not treated with aminoglycosides or tetracyclines, but the presence of pUK2002 and pUK2003 suggests that the spread of plasmids or resistant strains resulted from co-selection by tetracycline or streptomycin usage. pUK2001 only carried apramycin resistance, and we hypothesize that some factor, other than antibiotic use, is responsible for the selection and spread of this plasmid. The selective force may be the use of disinfectants or feed supplements on the farm, and work is currently underway to investigate these hypotheses. The presence and spread of apr R plasmids in commensal E. coli, under unknown selective pressures, poses severe implications for the transmission of this resistance determinant into clinical bacteria.

Acknowledgements

This work was funded by a veterinary faculty scholarship at the University of Edinburgh.

This study is a component of the International Partnership Research Award in Veterinary Epidemiology (IPRAVE) www.vie.gla.ac.uk/wiprave, funded by the Wellcome Trust, (grant number GR068596/A/02/Z).

References


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