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Persistence of a wild type *Escherichia coli* and its multiple antibiotic-resistant (MAR) derivatives in the abattoir and on chilled pig carcasses

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ABSTRACT

The aim of this study was to evaluate the ability of an *Escherichia coli* with the multiple antibiotic resistance (MAR) phenotype to withstand the stresses of slaughter compared to an isogenic progenitor strain. A wild type *E. coli* isolate (345-2RifC) of porcine origin was used to derive 3 isogenic MAR mutants. *Escherichia coli* 345-2RifC and its MAR derivatives were inoculated into separate groups of pigs. Once colonisation was established, the pigs were slaughtered and persistence of the *E. coli* strains in the abattoir environment and on the pig carcasses was monitored and compared. No significant difference ($P>0.05$) was detected between the shedding of the different *E. coli* strains from the live pigs. Both the parent strain and its MAR derivatives persisted in the abattoir environment, however the parent strain was recovered from 6 of the 13 locations sampled while the MAR derivatives were recovered from 11 of 13 and the number of MAR *E. coli* recovered was 10-fold higher than the parent strain at half of the locations. The parent strain was not recovered from any of the 6 chilled carcasses whereas the MAR derivatives were recovered from 3 out of 5 ($P<0.001$). This study demonstrates that the expression of MAR in 345-2RifC increased its ability to survive the stresses of the slaughter and chilling processes. Therefore in *E. coli*, MAR can give a selective advantage, compared to non-MAR strains, for persistence on chilled carcasses thereby facilitating transit of these strains through the food chain.

1. Introduction

It is generally accepted that the use of antimicrobial agents in livestock production contributes to the increased incidence of antibiotic resistance in both commensal bacteria and pathogens (Aarestrupp et al., 2008b; Hammerum and Heuer, 2009). In 2008 59% of combined species antimicrobials sold for food animal production in the UK were authorised for pigs and/or poultry and the largest percentage of single species products was sold for use in pigs (http://www.vmd.gov.uk/General/DARC/pubs.htm). A number of studies using a pig model have demonstrated that antimicrobial treatment regimes commonly used in the industry are responsible for increasing the number of antibiotic-resistant enteric and zoonotic bacteria in pigs (Aarestrupp et al., 2008a; Delsol et al., 2003; Delsol et al., 2004a, b). These results provide strong evidence that treated pigs have the potential to enter abattoirs with a higher number of resistant bacteria than untreated pigs, thereby increasing the risk of such bacteria moving through the food chain and infecting man. Enne et al. (2008) showed that of 2480 *Escherichia coli* isolated from pigs at slaughter 92% were resistant to at least one antimicrobial and 62.8% were resistant to 3 or more unrelated antimicrobials from classes used in human medicine.

Multi-drug resistance in bacteria is commonly attributed to mobile genetic elements such as plasmids or transposons (Gold and Moellering, 1996; Jacoby and Archer, 1991). However chromosomal multi-drug resistance systems such as the multiple antibiotic resistance (*mar*) locus of *E. coli* may also be involved (Cohen et al., 1989; George and Levy, 1983). The *mar*-locus of *E. coli* is reported to mediate reduced susceptibility (4 to 8 fold) to a number of unrelated antimicrobials primarily by up-regulating the outflow of antimicrobials via the AcrAB-TolC efflux pump (Okusu and Nikaido, 1996) and down-regulating influx through the Outer Membrane Protein F (OmpF) (Cohen et al., 1989). The up-regulation of the *soxRS* regulon has also been shown to up-regulate *acrAB*, resulting in the MAR phenotype (Miller et al., 1994; White et al., 1997).

The MAR phenotype confers low level resistance to antibiotics such as β-lactams, tetracyclines and fluoroquinolones, resistance to organic solvents such as cyclohexane (White et al., 1997) and decreased susceptibility to disinfectants such as chlorhexidines, acridines and triclosan (Mc Murray et al., 1998; Moken et al., 1997; Russell, 2000,

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2002) despite these products having diverse intracellular targets. Whilst the level of resistance is low, data suggests that it acts as an intermediate step towards higher levels of antimicrobial resistance (Alekshun and Levy, 1997; Levy, 2000) possibly providing the organisms with a competitive advantage in environments where antimicrobials and disinfectants are used. Other studies have also identified an association between fluoroquinolone resistance and organic solvent tolerance both in human clinical *E. coli* isolates (Oethinger et al., 1998) and in pig *Salmonella enterica sv*. Typhimurium DT104 isolates (Delsol et al., 2004a, b). In fact the latter study also demonstrated that pigs treated with a fluoroquinolone were entering abattoirs with an increased number of organic solvent-resistant bacteria compared to untreated pigs.

In order for antimicrobial-resistant bacteria to pass through the food chain, these bacteria must survive as contaminants during slaughter, food processing and retailing. Data generated during the course of previous studies indicates that following antimicrobial treatment, bacteria expressing the MAR phenotype may be better able to survive certain stresses, such as disinfection, that occur during food processing (Moken et al., 1997; Randall and Woodward, 2001; Russell, 2000, 2002). If so, MAR strains may have a greater potential to pass along the food chain to humans. In this study we evaluated the ability of *E. coli* expressing the MAR phenotype to withstand the stresses of slaughter and food processing compared to an otherwise isogenic parent strain and hence whether such MAR mutants are more likely to be transmitted through the food chain than wild type bacteria.

2. Materials and methods

2.1. E. coli strains

A wild type *E. coli* (345-2RifC) isolate of porcine origin marked with a no-cost chromosomal rifampicin-resistance mutation, as determined by assays to measure competitive fitness, was used for this study (Enne et al., 2005). This isolate was chosen as it had previously been used in the same animal model and had shown excellent colonisation characteristics (Enne et al., 2005).

MAR mutants were derived by passaging 345-2RifC in the presence of tetracycline (5 mg/L) at 30 °C as previously described (George and Levy, 1983; Randall and Woodward, 2001). Colonies were selected and passaged a second time in the presence of tetracycline (5 mg/L) to ensure purity, before storing on cryogenic beads at −80 °C. A total of 20 mutants were tested for their suitability in animal studies.

The minimum inhibitory concentration (MIC) values of ampicillin, chloramphenicol, nalidixic acid and tetracycline were determined for the parent strain (345-2RifC) and its MAR derivatives using the method of the British Society for Antimicrobial Chemotherapy (Andrews, 2001). Resistance to cyclohexane was tested as previously described (Randall et al., 2001). The susceptibility of the MAR mutants to ampicillin and chloramphenicol and their tolerance to cyclohexane were re-determined after five passages in Luria–Bertani (LB) broth (VWR, Pennsylvania, USA; Becton-Dickinson and Company, New Jersey, USA) to establish the stability of mutants.

Growth curves were performed in LB broth and minimal medium over a 24-h period for the parent strain and stable mutants. Inocula of the strains were grown overnight at 37 °C in the respective media (LB or minimal medium) and then the main culture inoculated at 10^5 CFU/mL in 4 replicate wells of a 96 well micro-titre plate and incubated at 37 °C. The optical density was recorded at intervals of 15 min for the first 4 h and then every 30 min up to 24 h. The optical density was recorded using a FLUROstar OPTIMA plate reader (BMG Labtech, Aylesbury, UK) set to read in absorbance mode at 600 nm.

To identify mutations in the mar-locus of MAR mutants, PCR amplification of the *acrR*, *marR* and *soxR* genes and their respective promoter regions was performed using the primers listed in Table 1 and an "Expand High Fidelity PCR System" (Roche Molecular Diagnostics, California, USA) according to the manufacturer's instructions. The PCR thermocycler conditions were as follows; 94 °C for 5 min; 30 cycles of 30 s denaturing at 94 °C, 1 min annealing at 50 °C and 1 min extension at 72 °C; followed by a final extension of 72 °C for 10 min, and then holding at 4 °C. PCR amplicons were sequenced using an ABI PRISM 377 automated DNA sequencer (Applied Biosystems, California, USA). The DNAstar LaserGene SeqMan program was used to assemble sequences and to compare them to the relevant accession numbers and parent strains.

2.2. Pig model

For each investigation, six 5-month old pigs from one litter (Large White cross) were housed as 2 groups of 3 in separate pens with HEPA filtration. The pigs were fed, *ad libitum*, standard grower rations on which they had been fed since weaning. All reached slaughter weight within 2 weeks of their inclusion in the study.

All animal procedures complied with Animal (Scientific Procedures) Act 1986 and were performed under Home Office Licence.

For the inoculation in the pig model, the parent strain and the MAR derivatives were grown separately overnight in nutrient broth (Oxoid Limited, Basingstoke, UK) at 37 °C. The cells were centrifuged at 5000 g for 15 min, washed in saline and re-suspended in antacid composed of $4MgCO_3.Mg(OH)_2.4H_2O$ (50 g/L); $Mg_2Si_3O_8$ (50 g/L) and $NaHCO_3$ (50 g/L). Bacterial/antacid suspension (50 mL) containing 10^9 CFU of culture was administered to each pig by oral gavage within 30 min of preparation. The suspension for the MAR derivatives was composed of

Table 1
Primers used to produce PCR amplicons for initial and subsequent sequencing.[a]

Forward primer	Sequence (5′ to 3′)	Reverse primer	Sequence (5′ to 3′)	Amplified base pairs
Accession number U00734				
*acrRO*F	GAACCTGAAGAACGACCTGA	*acrRO*R	CATCAGAACGACCGCCAGAG	197–1140
*AcrRO*1F	GATTACGTTGTGCCTGTTGC	*AcrRO*1R	ACATCCTCGATGTGGCTCTA	
*AcrRO*2F	GACGTAGAGCCACATCGAGG			
Accession number M96235				
*marRO*F	CCAGCCCCAGGCCAATTGC	*marRO*R	CGCTTGTCATTCGGGTTCG	1200–1726
*marRO*1F	CGTGGCATCGGTCAATTCAT	*marRO*1R	GGATAGAGCAGAGCACTTA	
*marRO*2F	TGTCTCCGCTGGATATTACC	*marRO*2R	CTGGACATCGTCATACCTCT	
Accession number X59593				
*soxRO*F	TAAGCGGCTGGTCAATATGC	*soxRO*R	AATGAGGTGTGTTGACGTCG	614–1329
*soxRO*1F	GGCAATCAGCGGCGATATAA	*SoxRO*1R	CGGAATGCCAATACGCTGAG	
*soxRO*2F	CAATGGCGAGAAGAGTTGGA	*SoxRO*2R	AATGCGCCGATCCAACTCTT	

[a] Primers underlined are primers to amplify the entire region. These primers and other primers were subsequently used in sequencing reactions.