Mechanisms of resistance in Salmonella enterica.

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Summary: The aim of this study was to promote resistance in Salmonella enterica serovar Enteritidis, Typhimurium, and Virchow to commonly used antibacterials and to identify mechanisms underlying any resistance obtained. Strains were exposed to various biocides and following each passage adaptive resistance was recorded. Permeability changes in the outer membrane, including LPS, cell surface charge and hydrophobicity and efflux were investigated as possible resistance mechanism candidates. The outer membrane and LPS bands were analysed by SDS-PAGE and visualised by Coomassie blue and silver staining. The cell surface charge and hydrophobicity were investigated employing microelectrophoresis and microbial adhesion to hydrocarbons assay, respectively. Efflux activity was examined by comparing resistance in pre- and post-adapted strains in the presence of reserpine. The outer membrane and LPS did not reveal any significant changes. Most of the parent strains were not significantly hydrophobic, whereas adapted were. An active efflux system was associated with benzalkonium chloride and chlorhexidine resistance.

Keywords: Adaptive resistance, Salm. Enteritidis, Salm. Virchow, Antibiotics, Biocides.

Introduction: Bacteria continue their natural evolution and can develop resistance mechanisms to antibacterial drugs at an incredible speed. This has created a growing public health problem, which may be exacerbated by the commonplace and often unnecessary addition of biocides into household products. Antimicrobial resistance among human and veterinary bacterial pathogens develops through one or more several possible mechanisms including permeability changes in the cell membrane, active efflux, enzymatic inactivation or destruction of the antimicrobial, alteration of the target site of antimicrobial action and creation of altered enzymatic pathways among others. The majority of antimicrobials used in the veterinary medicine can be blocked by one of these or a combination of these mechanisms (White, 2000). Thus, this study was aimed to understand how bacteria manage to endure antimicrobial action by investigating the mechanisms underlying resistance.

Materials and Methods: Antimicrobial susceptibility testing, the reserpine effect and bacterial adaptation: Standard broth dilution method was carried out using a two-fold dilution of each antibacterial agent (Loughlin et al., 2002) and reserpine for the MIC determination of approximately 10^8 bacteria. The MICs of pre- and post- adapted Salmonella enterica cultures were also obtained in the presence and absence of reserpine 20Ìg/ml together with the antibacterials. After the overnight incubation period at 37 °C bacterial growth was assessed by observing turbidity in the media. Bacterial adaptation was performed according to the technique described by Joynson et al., 2002. Following adaptation, random amplification of polymorphic DNA (RAPD) was employed to confirm the molecular identity of the strains. (Hopkins and Hilton, 2001).

Preparation and analysis of outer membrane proteins (OMP) and lipopolysaccharide (LPS): Outer membrane extracts were prepared by a method based on Lambert et al., 1982. Each membrane suspension (30Ìl) in loading buffer was electrophoresed at 200 V for 42 minutes in an 11% polyacrylamide gel using Bio-Rad Mini Protean II apparatus. The LPS bands were visualised either by silver staining (Fomsgaard et al., 1990) or by Coomassie blue stain. Cell Surface Hydrophobicity (CSH) & Charge: The cell surface hydrophobicity of Salmonella enterica strains was determined by microbial adhesion to hydrocarbon (MATH) assay employing n-hexadecane...
as the hydrocarbon phase (Loughlin et al., 2002). The cell surface charge was determined by particle microelectrophoresis using a Zetamaster Particle Electrophoresis Analyser, (Brookhaven Instruments) to measure the zeta potential. Bacterial cells were grown in nutrient broth to stationary phase and suspended in 1mM (KCL) at a concentration of approximately 1x10^7 cells/ml.

Statistical Analysis: The data for MATH and microelectrophoresis were checked for normality using the Kolmogorov-Smirnov test. The MATH and microelectrophoresis results were statistically analysed using a single factor analysis of variance (ANOVA) and Fisher LSD post hoc analysis. All analysis was carried out using the Statistica Program.

Results: Antimicrobial Susceptibility Testing, the Reserpine Effect and bacterial adaptation: Resistance was obtained in all strains investigated within 6 days of gradual exposure and strain continuity was confirmed by RAPD profiling (Figure 1). Results strongly indicated the operation of an efflux pump system in benxzalkonium chloride (BKC) and chlorohexidine (CHX) resistance (data not shown).

Preparation and analysis of outer membrane proteins and LPS: No significant difference in outer membrane and LPS profiles were apparent between pre- and post-adapted strains. Figure 2a represents TLN-adapted Salmonella Virchow by silver staining and figure 2b shows pre-and post-adapted Salm. Virchow strains visualised by Coomassie brilliant blue.

Cell surface Hydrophobicity and Charge: Benzalkonium chloride adapted-Salm. Enteritidis, Salm. Typhimurium and Salm. Virchow, as well as erythromycin-adapted Salm. Enteritidis and Salm. Typhimurium showed significant changes in hydrophobicity. More specifically, parent strains did not reveal any significant hydrophobicity, whilst adapted did (data not shown). All strains investigated shown to carry a negative charge. No significant changes between pre- and post- adapted strains were apparent (data not shown).

Discussion: The presence of an active efflux pump, the outer membrane and LPS bands and the cell surface hydrophobicity and charge were examined as possible candidates of resistance. Efflux pumps play a vital role in the establishment of resistance of Gram-negative bacteria to a panel of antimicrobial agents (Rosenberg, 2000). More specifically, it was found that adapted strains returned to their parent MIC in the presence of reserpine, which consequently suggests the up-regulation of an efflux protein. In this study, there was a strong indication of BKC and CHX efflux mediated resistance. Resistance to BKC mediated by efflux pumps has been previously documented by Aase et al., 2000.
The mechanisms of CHX resistance remain unclear. Fang et al., 2002 proposed the possibility of a cationic efflux pump in CHX-adapted E. coli. Lipopolysaccharides are the main components of the outer membrane of Gram-negative bacteria and are responsible chiefly of the cell impermeability characteristics. Outer membrane and LPS profiles did not reveal any significant changes in all Salmonella enterica strains investigated. This is rather unusual since a number of reports support that resistance in Gram-negative bacteria might be associated with changes in outer membrane including LPS (Louglin et al., 2002). One of the perceptible effects of the biocidal interaction with the bacterial cell is a change in cell surface hydrophobicity (Maillard, 2002). In this study, Salm. Enteritidis, Salm. Typhimurium and Salm. Virchow adapted to BKC, as well as Salm. Enteritidis and Salm. Typhimurium adapted to ERY were significantly more hydrophobic than the parents. In addition there was no significant difference in the cell surface charge between parent and adapted strains and no correlation between hydrophobicity and charge was evident as proposed by Wilson et al., 2001.

Conclusions: In summary, increased cell surface hydrophobicity and the presence of an active efflux pump could facilitate the acquisition of antibacterial resistance in Salmonella enterica, providing cross-resistance to a range of antibiotics and biocides. The emergence of bacterial antimicrobial resistance might be associated with the imprudent use of antibacterials in agriculture. White (2000) proposed that pork producers and veterinarians must treat ill animals; however misuse of antimicrobials in swine could negatively influence consumer confidence in pork products.

References: