Antibiotic resistance in the environment, with particular reference to MRSA

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I. Introduction

The introduction of β-lactam antibiotics (penicillins and cephalosporins) in the 1940s and 1950s probably represents the most dramatic event in the battle against infection in human medicine. Even before widespread global use of penicillin, resistance was already recorded. *E. coli* producing a penicillinase was reported in Nature in 1940 (Abraham, 1940) and soon after a similar penicillinase was discovered in *Staphylococcus aureus* (Kirby, 1944). The appearance of these genes, so quickly after the discovery and before the widespread introduction of penicillin, clearly shows that the resistance genes pre-dated clinical use of the antibiotic itself.

Intuitive reasoning would suggest that antibiotic resistance occurs due to direct selection produced by the use of antibiotics in humans and animals. For example, the mutations associated with increased resistance to fluoroquinolones have been documented in specific regions of the *gyrA, gyrB, grlA*, and *grlB* genes, which are referred to as the quinolone resistance-determining regions (QRDRs) (Piddock, 1998). Selection for resistance to a given antibiotic may take place within an infected human treated with antibiotics. However, selection may occur in other environments such as waste water treatment systems, agricultural environments where antibiotics may be of veterinary origin or within an environmental background where antibiotic selection is provided by bacterial antibiotic producers.

In contrast to the scenario where resistance is conferred by mutation and selection by medical antibiotics, resistance can occur in an organism by the acquisition of a novel gene. New genes are acquired by horizontal gene transfer (HGT), either through conjugation, transformation or transduction. The origins of mobile antibiotic resistance genes may be from bacteria which have been subject to antibiotic selection in a nosocomial environment, or from environmental bacteria.

An example of an environment where HGT is likely to occur is soil. Practices such as sewage sludge and animal slurry application to land introduce complex mixtures of bacteria containing drug resistance genes, medical and veterinary antibiotics and other chemicals such as detergents and surfactants to land where interactions may occur with indigenous soil bacteria (Figure 1).

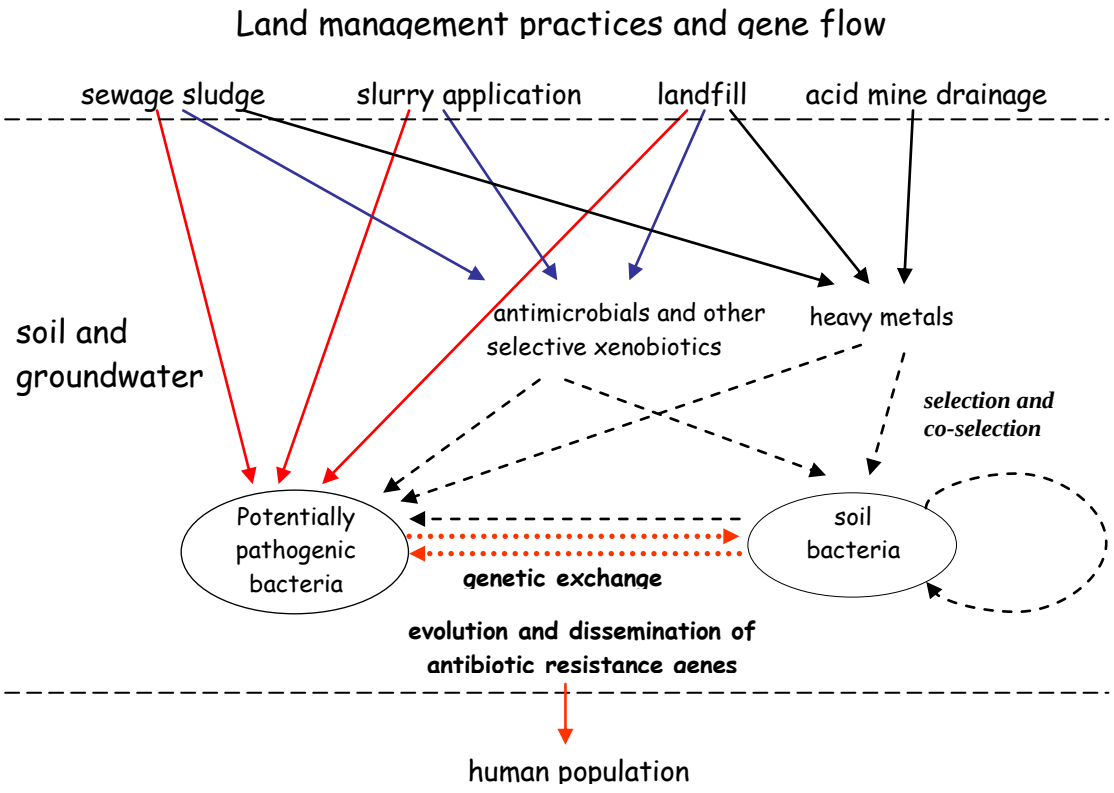

Figure 1. Anthropogenic sources of bacterial pathogens, pharmaceuticals and heavy metals which in conjunction with indigenous soil bacteria provide a mixture of genes and selective pressure for selection or co-selection of antibiotic resistance.

II. Evolution of resistance

Antibiotic resistance has two components, the evolution of genes with novel activities and the evolution of mechanisms allowing horizontal transfer throughout the microbial population.

A. Origins of antibiotic resistance genes

Many modern β-lactam antibiotics such as the 7-α-methoxycephalosporins are secondary metabolites of *Streptomyces* species, the majority of streptomycetes and other actinobacteria produce constitutively expressed β-lactamases which have a high GC content (≥70%). However, the β-lactamases encountered in human and animal pathogens all have GC contents in the order of 45-65% suggesting a Gram-negative origin. There are a number of Gram-negative bacteria which exist in close proximity to antibiotic producers in the rhizosphere, which would require the capability of protecting themselves against the toxic metaboloites of their neighbours. Certain plant pathogens and rhizobacteria such as *Erwinia, Serratia, Flavobacterium, Pseudomonas, Chromobacterium* and *Agrobacterium* sp. produce carbapenems, β-lactams and moncocyclic β-lactams (Jensen and Demain, 1995).

The rhizosphere is the soil compartment influenced by plant root metabolism, and is high in nutrients derived from root exudates consisting of compounds such as organic acids, sugars, amino acids, vitamins and carbohydrates. The rhizosphere is an important niche for microorganisms which are involved in nutrient recycling and plant health. The origin of resistance genes in the rhizosphere is likely to result from competition between microrganisms for colonisation sites. Key mechanisms responsible for selection of medically significant bacteria in the rhizosphere are discussed in a recent review (Berg et al., 2005). Many mechanisms involved in the interaction between antagonistic plant-associated bacteria and their host plants are similar to those responsible for bacterial pathogenicity including pathogenicity in humans (Rahme et al., 1995).

It has long been suspected that the environment constitutes a reservoir of novel antibiotic resistance genes, although its significance has been overlooked in favour of

evolution of resistance within the clinical environment. Arguably one of the most clinically important groups of β-lactamases in Gram negative bacteria at the moment are the CTX-M family (Livermore and Hawkey, 2005). The identification of environmental progenitors of the extended-spectrum β-lactamase (ESBL) CTX-M enzymes responsible for resistance to 3rd generation cephalosporins (3GCs) in bacteria of the genus *Kluyvera* clearly indicates the significance of the environment in the evolution of emerging antibiotic resistance determinants (Bonnet, 2004; Rodriguez et al., 2004). *Kluyvera* sp. are rare human pathogens causing similar infections to *E. coli* and are more often found associated with plants. *K. ascorbata* has been shown to enhance plant growth, particularly in heavy metal contaminated soils (Burd et al., 1998; Burd et al., 2000). *K. georgiana* produces KLUG-1 the nucleic acid sequence of which clusters with the CTX-M-8 group. Sequence similarity between the genes suggests that the natural β-lactamases of *K. ascorbata* and *K. georgiana* are the progenitors of the CTX-M-2 and CTX-M-8 groups respectively (Bonnet, 2004). Evidence suggests that the process of gene transfer from the chromosome of *Kluyvera* to other clinically important bacteria has occurred several times involving different mobile elements, such as the IS-10-like element found upstream of both KLUG-1 and CTX-M-8 and ISEcp1 found upstream of KLUA-1 and members of the CTX-M-2 group (Poirel et al., 2001). ESBLs confer low level resistance to β-lactams, *K. cryocrescens* possessing the KLUC-1 ESBL only conferred resistance to cefotaxime, ceftriaxone, cefpirome and aztreonam when cloned into *E. coli* (Decousser et al., 2001). It is probable that KLUC-1 is only weakly expressed in *K. cryocrescens*, but mutations in the promoter region would confer ESBL resistance. Biochemical analysis of KLUC-1 revealed that substrate specificity and substrate profile are similar to those reported for CTX-M enzymes.

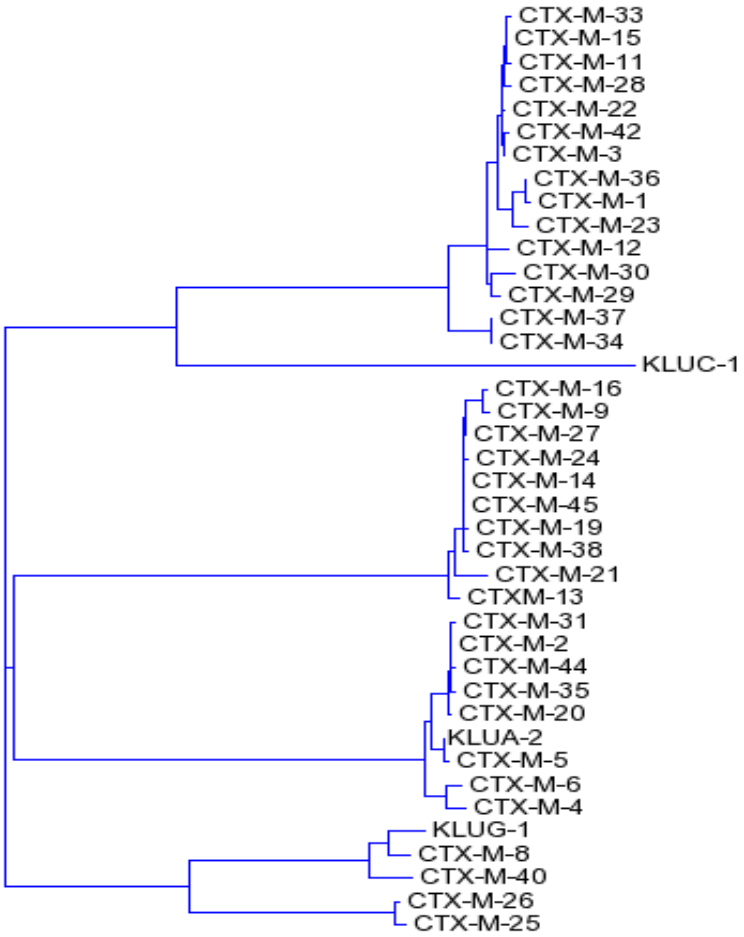

Figure 2. Unrooted tree illustrating the phylogenetic relationships of KLUC-1, KLUG-1 and KLUA-1, Kluyvera sp. chromosomal genes which are the putative progenitors of the CTX-M-1, CTX-M-8 and CTX-M-2 groups of ESBL enzymes (neighbour joining tree, Trex).

ESBLs confer resistance to 3GCs which are semi-synthetic molecules that do not exist in nature as far as is known, their structure is however basically that of a cephalosporin of which a number of naturally produced compounds exist in the environment. Very little is understood about the prevalence, ecological function and diversity of ESBL genes in the horizontal gene pool in soil, whether they are in

transiently resident human/animal derived bacteria, or in the chromosomes of bacteria permanently residing in the rhizosphere.

A fluoroquinolone resistance gene *qnrA* has recently been established as originating in the water-borne species *Shewanella algae*, this genetically mobile plasmid-borne gene has now moved into clinically important bacteria and again shows the importance of the natural environment as a reservoir of clinically important antibiotic resistance genes (Nordmann and Poirel, 2005). The *qnrA* genes are embedded in complex sul1-type integrons which also carry CTX-M-2 and CTX-M-9 ESBLs.

B. Mechanisms of resistance

There are four main types of antibiotic resistance in bacteria (Hawkey, 1998). Antibiotic modification allows retention of the same target as sensitive strains, but the antibiotic is modified before it reaches the target. β-lactamases enzymatically cleave the β-lactam ring, inactivating the antibiotic. Some bacteria stop the antibiotic entering the cell or by efflux, pumping the compound out of the cell. Carbapenem β - lactam antibiotics enter Gram negative bacteria via a membrane protein known as a porin, resistant bacteria can lack the specific D2 porin responsible for transport and are therefore resistant (Pirnay et al., 2002). Efflux via a membrane pump is a common mechanism and is found in Gram negative and Gram positive bacteria where five different superfamilies of efflux pumps conferring antibiotic resistance have been reported (Mahamoud et al., 2007) .

Changes in the target site also produce resistance; the antibiotics are able to reach the target but are not able to inhibit the target due to changes in the molecule. Enterococci are regarded as inherently resistant to cephalosporins because the

enzymes responsible for cell wall synthesis (peptidoglycan production), known as penicillin binding proteins (PBPs), have a low affinity for cephalosporins and are not inhibited (Hawkey, 1998). Resistance to β-lactam antibiotics in pneumococci is due entirely to the development of altered forms of the high-molecular-weight (PBPs) that have decreased affinity for the antibiotics (Coffey et al., 1995). Altered PBPs have emerged by recombinational events between the pbp genes of pneumococci and their homologs in closely related streptococcal species.

The fourth antibiotic resistance mechanism (usually an enzyme) is the production of an alternative target that is resistant to the antibiotic, whilst continuing production of the sensitive target. Methicillin resistant *Staphylococus aureus* (MRSA) produces an alternative penicillin binding protein (PBP2a) which is encoded by *mecA* carried on the Staphylococcal Cassette Chromosome mec (SCCmec). Because PBP2a is not inhibited by the antibiotics, the cell continues to produce peptidoglycan and maintains a stable cell wall (Hardy et al., 2004b)

III. Mechanisms of horizontal gene transfer

Gene transfer in the environment is central to the hypothesis that a reservoir of novel resistance genes exists out side the clinic, which can be transferred to clinically significant bacteria in the clinic. An extensive literature on genetic exchange between bacteria in the environment exists which is reviewed elsewhere (Davidson, 1999). The current review concentrates specifically on gene transfer mediated by transposable elements such as class 1 integrons which are of increasing clinical importance. The occurrence of co-selection by non-antibiotic xenobiotics is also discussed.

The evolutionary response of bacteria exposed to antibiotics has been mediated in large part by the movement of conjugative plasmids carrying

antimicrobial resistance genes in both Gram negative and Gram positive bacteria. Transfer frequencies for conjugative plasmids in *Enterococcus faecium* can be as low $\geq 10^{-6}$ per donor in laboratory experiments using broth mating, but the production of short peptides by some recipient cells which cause cell aggregation greatly increase transfer rates ($\geq 10^{-4}$) (Dunny et al., 2001). Plasmids play an important role in gene transfer in staphylococci, conjugative plasmids also being capable of mobilising small non-transferable plasmids (McDonnell et al., 1983). Gene transfer does not always require the presence of plasmids as the extensively studied and widely distributed conjugative transposons such as Tn916 demonstrate (Burrus and Waldor, 2004). The gut of both humans and food animals is probably the most important site for transfer of conjugative plasmids as careful studies have shown there is no loss of fertility of conjugative plasmids in the gut and conjugation most probably occurs continuously in the gut of humans/animals even in those not receiving antibiotics (Freter et al., 1983). The release of faeces into the environment represents probably the most important source of novel assortments and types of resistance genes in new bacterial hosts. The laboratory simulation of plasmid transfer in the environment can give low transfer frequencies, but these must be compared to the size of the populations involved. The requirement for close contact in the laboratory (e.g. filter mating) may readily be met in microenvironments: staphylococci on the skin, oral streptococci in dental plaque or pseudomonas in water films on soil particles.

A. The role of integrons in resistance gene mobility

In addition to multi-resistance plasmids antibiotic resistance genes are situated on transposable elements that can associate with other elements such as chromosomes, and plasmids. These transposable elements include transposons and integrons, which

can be transferred horizontally. Integrons are recombination and expression systems that capture genes as part of a genetic element known as a gene cassette (Recchia and Hall, 1995). Gene cassettes bear a recombination site known as a 59-base element (59-be) that is recognised by the integron-encoded integrase IntI (Hall et al., 1991). Most cassette genes described are antibiotic or quaternary ammonium compound resistance genes. However, recent studies have revealed that the cassette gene pool is far more diverse than previously thought. Stokes et al. (2001) designed PCR primers to conserved regions within the 59-be of gene cassettes, allowing detection of a large number of novel genes. Using these primers, one hundred and twenty three cassette types were recovered from Antarctic and Australian soils and sediments, with very few represented in clone libraries more than once indicating the large size of the cassette gene pool. Most ORFs did not match known sequences, again illustrating the diversity of these gene sequences. Further studies revealed an additional 41 environmental gene cassettes, giving a total of 164 directly sampled from natural environments by PCR (Holmes et al., 2003). There are several classes of integrons, the most commonly studied being class 1 integrons that are commonly associated with antibiotic resistant bacteria. Recent studies have detected novel integron classes in soils, Nield et al. (2001) classified three new integron classes and Nemergut et al. (2004) an additional 14 classes, demonstrating the immense variety of these elements present in the environment.

The variable regions of class 1 integrons contain the cassette genes, and to the right of this lies the 3'-conserved region which may have one of three different backbone structures (Partridge et al., 2002). The first backbone type consists of a Tn402 (In16) like arrangement consisting of a *tni* module containing 3 transposition genes and a resolvase gene, the second In5 type consists of *qacE*Δ1, sul1, orf5, orf6 a

partial *tni* module *tni*Δ consisting of two transposition genes. The third In4 type just carries *qacE*Δ1, sul1, orf5 and orf6. Integrons carrying the complete *tni* module are able to undergo self-transposition and it is thought that In5 and In4 types may also be able to move if the *tni* gene products are supplied in *trans* (Partridge et al., 2002).

The role of class 1 integrons in conferring antibiotic resistance to clinical isolates of many bacterial strains is well documented (Briggs and Fratamico, 1999; Leverstein-van Hall et al., 2003; Segal et al., 2003; White et al., 2000). Fluit and Schmitz (2004) summarised recently described cassette gene diversity which include 25 β-lactam resistance genes including 8 carbapenemase and 17 ESBL genes, 11 aminoglycoside, 2 chloramphenicol, rifampicin, 3 trimethoprim and quinolone resistance genes. It is therefore clear that integrons are capable of conferring resistance to extended spectrum β-lactams, carbapenems and fluoroquinolones, representing an extremely efficient method of acquiring resistance to the most widely used and important antibiotics. Studies on the incidence of class 1 integrons in bacterial pathogens associated with agriculture and fish farming such as *E. coli* and *Aeromonas salmonicida* have shown a link between integrons and antibiotic resistance (Bass et al., 1999; Sorum et al., 2003). A recent study (Nemergut et al., 2004) has shown evidence of a gene cassette encoding nitroaromatic catabolism, a group of compounds associated with mining activity, this highlights the fact that other selective pressures other than antibiotics may also co-select for resistance genes. The process by which a gene becomes a movable cassette is not understood; however, it has been proposed that the 59-base element is added as a transcription terminator to an RNA gene transcript that is subsequently converted into DNA by a hypothetical reverse transcriptase (Recchia and Hall, 1995).

Class 1 and 2 (also capable of carrying antibiotic resistance genes) integrons are known to undergo transfer between bacteria in chicken litter, which is often spread onto soil where further selection and horizontal transfer may occur (Nandi et al., 2004). The later study was the first to demonstrate wide spread prevalence of class 1 integrons in Gram positive bacteria, illustrating the potential for HGT.

B. Co-selection for resistance genes

It is commonly assumed that in the absence of antibiotic selection, mobile resistance genes will be lost, and the host return to a sensitive phenotype as the genes confer a resistance cost on the host. Co-selection is one mechanism, whereby other resistance genes carried on the same genetic element produce selection for an entire mobile genetic element. Anthropogenic activity produces emissions of complex mixtures of xenobiotics, bacterial pathogens and antibiotic resistance genes into the environment, in the form of industrial and domestic effluent, human and animal waste. Industrial and domestic pollutants such as quaternary ammonium compounds (QACs) have been shown to exert an extremely strong selective pressure for class 1 integrons which are a major mechanism for dissemination of antibiotic resistance (Gaze et al., 2005). Co-selection is produced by the presence of QAC resistance genes on multi-resistance plasmids or class 1 integrons. QAC resistance genes fall into two families; *qacA/B* belong to the Major Facilitator Superfamily and are only found in staphylococci on multi-resistance plasmids (Paulsen et al., 1996). Other QAC resistance genes belong to the Small Multidrug Resistance Family and include *qacC/D* now known as *smr*, *qacE, qacEΔ1, qacF, qacG, qacH* and *qacJ* (Gaze et al., 2005). *QacE, qacEΔ1, qacF* and *qacG* have been identified on integrons and the remaining genes on multi-resistance plasmids in staphylococci. In a study investigating QAC pollution and class

1 integron prevalence, bacteria were isolated from a reed bed used to remediate effluent from a textile mill (Gaze et al., 2005). QAC resistance was higher in isolates from reed bed samples and class 1 integron incidence was significantly greater in populations pre-exposed to QACs.

Exogenous plasmid isolation has been used to detect resistance genes in soil bacteria. This method allows capture of plasmids from the total bacterial fraction of an environmental sample without the necessity to culture the host organism. Smit and colleagues (Smit et al., 1998) investigated mercury resistance plasmids in soil populations using exogenous isolation, and identified plasmids of 10-50kb carrying resistance to copper, streptomycin and chloramphenicol. These authors amended soil with mercuric chloride and found this to subsequently increase the recovery of resistance plasmids, highlighting the fact that heavy metals may co-select for antibiotic resistance in the environment. Plasmids have also been captured from polluted soils and slurrys (Smalla et al., 2000; Top et al., 1994). The latter authors identified multiple antibiotic resistance genes from isolated plasmids. Some aspects of animal husbandry using heavy metal containing compounds select for antibiotic resistance genes in the environment, such an example being the use of copper growth supplements in pigs (Hasman et al., 2006). In Denmark glycopeptides were banned in food animal production in 1995, following that ban the rate of glycopeptide resistance in *Enterococcus faecium* (GRE) did not change. The banning of macrolides in 1998 led to a significant fall, this was thought to be due to both antibiotic resistance genes being on the same plasmid and co-selection by the continuing use of macrolides (Aarestrup, 2000). GRE continue to be prevalent in Danish pigs, the explanation is now thought to be the linkage on the same plasmid of the copper resistance gene *tcrB*

and macrolide/glycopeptide resistances. A feeding experiment using 175mg Cu/kg in feed selected for GRE in piglets, whereas 6mg/kg feed did not (Hasman et al., 2006).

IV. Antibiotics and resistance genes in the environment

Human and animal wastes may contain antibiotics or active intermediates from human and veterinary medicines which may potentially increase antibiotic resistance selection in soil, in addition to introducing pathogens which can exchange mobile genes with indigenous rhizosphere bacteria. Antibiotics retain their selective capabilities in the soil and are ultimately released to surface waters (Boxall et al., 2002). Certain plant pathogens and rhizobacteria such as *Erwinia, Serratia, Flavobacterium, Pseudomonas, Chromobacterium* and *Agrobacterium* sp. produce carbapenems, β-lactams amd moncocyclic β-lactams (Jensen and Demain, 1995). In addition, streptomycetes and fungi produce a wide range of antibiotics.

A. Sewage sludge

The 2001 UK Sewage Sludge Survey showed that an average of 1,072,000 tonnes of dry solids per annum was produced in the years 1998 to 2000. The UK Department for Food and Rural Affairs (DEFRA) state that conventionally treated sludge has been subjected to defined treatment processes and standards that ensure at least 99 per cent of pathogens have been destroyed. Enhanced treatment, originally referred to as "Advanced Treatment", describes treatment processes which are capable of virtually eliminating any pathogens (99.9999 per cent) which may be present in the original sludge. Conventionally treated sewage sludge cannot be surface spread if the land is intended for grazing, it must be deep injected into the soil and left for at least three weeks until it is grazed. Conventionally treated sewage can be applied to the surface

of grassland, or for forage crops such as maize, which will then be harvested (no grazing allowed within the season of application). If being applied to land growing vegetables at least 12 months must have elapsed between treatment and harvest. When using enhanced treated sludges farmers must wait at least three weeks before grazing animals or harvesting forage crops, and at least 10 months before harvesting fruit and vegetable crops grown in direct contact with the soil and normally eaten raw (http://www.defra.gov.uk/farm/waste/sludge/index.htm). It is clear that the potential for transmission of resistance genes to food animals and vegetable crops exists.

A hydraulic, biokinetic and thermodynamic model of pathogen inactivation during anaerobic digestion showed that a 2 log10 reduction in *E. coli* (the minimum removal required by the UK government for agricultural use of conventionally treated biosolids) is likely to challenge most conventional mesophilic digesters (Smith et al., 2005). UK regulations for pathogen removal are becoming more stringent, but the processes used to reduce bacterial indicator species numbers may have a quite different effect on resistance gene numbers. β-lactam and aminoglycoside resistance genes have been isolated by exogenous isolation from activated sewage in Germany, illustrating that final stage sludge is a source of antibiotic resistance genes (Tennstedt et al., 2005).

Crucially, although sewage sludge has been demonstrated to contain antibiotic resistance genes and pathogenic bacteria, the extent of this problem and the potential for transfer of resistance to soil bacteria and ultimately its effect on the human population is unknown. Sewage sludge also contains measurable concentrations of antibiotics which may continue to select for resistance in the soil. A study by Golet *et al.,* (2003) suggested that sewage sludge is the main reservoir of FQ residues from

waste waters and outlined the importance of sludge management strategies to determine whether most of the human-excreted FQs enter the environment. Field experiments of sludge-application to agricultural land confirmed the long-term persistence of trace amounts of FQs in sludge-treated soils and indicated a limited mobility of FQs into the subsoil. Where sewage treatment plants receive large amounts of effluent from hospitals, the problem of antibiotic residues and resistance genes in sewage sludge may be particularly significant. Persistence of FQs is particularly relevant as they appear to co-select for class 1 integrons and integron borne ESBL genes due to the fact that recently discovered quinolone resistance genes are situated in class 1 integron structures which also confer ESBL resistance (Nordmann and Poirel, 2005). Our own research has indicated an impressive reservoir of FQ resistance (QRDR mutations) in staphylococci associated with free range chicken farming (Hawes, 2004).

Recent studies in Portugal have identified β-lactamases, including TEM, IMP and OXA-2 derivatives, in aquatic systems and ESBL resistance genes in sewage sludge, which is spread to land in the UK and therefore has the potential to recycle resistance to the human food chain (Henriques et al., 2006). ESBL-producing Enterobacteriaceae were detected in five samples of human sewage in Spain (Mesa et al., 2006).

The human colon is the major reservoir of emerging opportunistic pathogens such as *E. coli*, *Klebsiella*, *Enterobacter* and *Acinetobacter baumanii* (Agustía, 2002; Fanaro et al., 2003; Hollander et al., 2001) and it is likely that these are food-derived in the community (Turtura et al., 1990). The distinction between food-borne commensals, pathogens and nosocomial pathogens is some what arbitrary, and many emerging nosocomial gram negative pathogens may be food borne, normally living in

the gut as commensals until the individual becomes immuno-suppressed or antibiotic resistance genes transfer from another organism. Recent research has revealed that soil, particularly the plant rhizosphere harbours diverse opportunistic human pathogens including *Acinetobacter baumanii, Aeromonas salmonicida, Burkholderia cepacia, Enterobacter agglomorans, Klebsiella pneumonia, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella typhimurium, Serratia* sp., *Staphylococcus aureus, Stenotrophomonas maltophilia* and others (Berg et al., 2005).

B. Farm animals

When soils are treated with manure both residues of antibiotics used as veterinary medicines and bacteria carrying genes conferring resistance are introduced into the soil and reach the food chain, e.g., via plant-associated bacteria (Witte et al., 2000). Some 461 tonnes (active ingredient) of antimicrobial therapeutics and growth promoters were sold for use in food animals in 2000 in the UK (National Office of Animal Health data), including tetracyclines (228 t), trimethoprim / sulphonamides (94 t), β-lactams (49 t), macrolides (41 t) and FQs (1 t). Some veterinary antibiotics are synthetic, so unlike those which are produced by soil bacteria, many cannot be broken down through normal processes, so may persist for a long time in soils; adsorption to soil particles and other surfaces allow accumulation of residual antibiotics to high concentrations (Kummerer, 2004).

It has been clearly established that the use of certain antibiotics in agriculture has contributed to the development of resistant bacterial strains in human infection, as evidenced by the work of Wolfgang Witte and others where vancomycin resistance genes in human *Enterococcus faecalis* isolates were traced to the use of avoparcin in pigs (Witte, 1997). As early as the 1970s Stuart Levy noted that oxytetracycline was a

major feed additive and that studies had shown there was a strong association between tetracycline resistance in isolates from livestock and animal workers (Levy et al., 1976). In 1969 the Swann committee report recommended that antibiotics used in human medicine should not be used as growth promoters. Only in the last few years have a number of antibiotics been banned, and clearly their use as growth promoters is inadvisable.

A mixture of the parent product and metabolites are excreted in faeces and urine. Excreta enter the farm environment directly in grazing animals and indirectly in intensively reared animals through application of slurry and manure. It is estimated that approximately 70 million tons of animal manure wastes are spread onto agricultural land per annum in the UK (Hutchison et al., 2004). In many river catchments the bulk of faecal coliforms are believed to be of agricultural origin. Chee-sanford *et al.* (2001) screened for eight *tet* genes in groundwater associated with swine production facilities. Tetracycline resistance genes were found as far as 250m downstream from waste lagoons, highlighting the danger posed by use of antibiotics in agriculture and the risk of contamination of drinking water with antibiotic resistant bacteria. In a different study a detection limit of 10^{2}-10^{3}copies of the *tet*(M) gene per gram was achieved using a nested PCR method with TC-DNA (Agerso et al., 2004). The gene was detected in farmland soil previously amended with pig slurry containing resistant bacteria; the number of positive samples from farmland soils one year after manure treatment was significantly higher than in samples of garden soil not treated with manure.

E. coli strains producing an extended spectrum β-lactamase (ESBL) (CTX-M-2) were recently isolated from cattle faeces in Japan (Shiraki et al., 2004). β-lactamase and ESBL's have also been detected in *E. coli* isolates from healthy chickens, food

and sick animals in Spain (Brinas et al., 2003a; Brinas et al., 2003b; Brinas et al., 2002). The use of extended-spectrum cephalosporins in chickens is very unusual, and the possibility of cross-selection with other antimicrobials used in poultry (such as sulphonamides and tetracyclines, among others) might explain this discovery. In a study of retail chicken breasts, quinolone resistant *E. coli* also producing CTX-M-2 were found in 5 of 10 samples produced in Brazil (CTX-M-2 is widely reported in human infection with *Salmonella* and *E. coli* from South America), whereas only 1 of 62 samples of UK produced chicken were positive for CTX-M-1 (Ensor, 2007) ESBLs were also discovered in samples from 8 of 10 pig farms, 2 of 10 rabbit farms, from all 10 poultry farms tested and in 3 of 738 food samples studied in Spain (Mesa et al., 2006).

The FQ antibiotic enrofloxacin (the major metabolite being ciprofloxacin) has been used extensively in poultry farming and evolution of fluoroquniolone resistance in chicken litter has been documented, caused by mutation in the quinolone resistance-determining region (QRDR) of the *gyrA* gene (Lee et al., 2005). Plasmid-mediated quinolone resistance (PMQR) is also known to occur. The gene responsible for PMQR has been identified as *qnr* and this gene was found in an integron-like element and is associated with the ESBL VEB-1 (Poirel et al., 2005c). The origin of *qnrA* has recently been established as the water-borne species *Shewanella algae* which, in addition to the fact that CTX-M-ases originate in the rhizosphere *Kluyvera sp.*, underlines the role of the environment itself as a reservoir of novel resistance genes (Nordmann and Poirel, 2005; Poirel et al., 2005b). Recently further transferable quinolone resistance genes *qnrB* and *qnrS* have been identified in gram negative opportunistic pathogens (Poirel et al., 2005a). In 2004, an entirely novel plasmid mediated mechanism of quinolone resistance was discovered. In *E. coli* from

Shanghai, PRC strains with a MIC of 1.0mg/L of ciprofloxacin carried a mutated form of the aminoglycoside inactivating enzyme AAC (6^1)-Ib-cr (Robicsek et al., 2006). This enzyme N-acetylates quinolones which have an amino nitrogen on the piperazinyl substituent (e.g. ciprofloxacin and norfloxacin). The distribution of the gene has not been studied extensively but has been found in China and North America (Robicsek et al., 2006). It has been recently reported to be associated with the ESBL gene $bla_{\text{CTM-M-15}}$ and $bla_{\text{OXA-1}}$ and $bla_{\text{TEM-1}}$ on a limited range of plasmids carried by E. coli in Portugal (Machado et al., 2006). The consequences could be grave as ciprofloxacin is extensively either used in agriculture or is the principal metabolite of veterinary quinolones such as enrofloxacin. The use of quinolones (which are also slow to degrade in the environment) will co-select for a wide range of *qnr/acc*(6^1) associated resistances such as resistance to 3rd generation cephalosporins and carbapenems. Thus, there is increasing evidence of an environmental reservoir of clinically important antibiotic resistance genes.

Several studies demonstrated a correlation between the extent of use of antibiotics in animals and the incidence of the respective antibiotic resistance genes. Thus it is not surprising to learn that animal manure, in particular piggery manure has been shown to be a "hot spot" (high incidence) of bacteria carrying antibiotic resistance genes residing on mobile genetic elements (Smalla et al., 2000).

Antibiotic resistance does not always appear to be directly related to short term trends in antibiotic useage. The glycopeptide growth promoter avoparcin was banned from animal production Denmark in 1995, and in the EU in 1997, due to concern for the spread of vancomycin-resistant enterococci (VRE) from food animals to humans. A Danish study found high levels of VRE in broiler flocks five years after avoparcin was withdrawn (Heuer et al., 2002a). Further studies revealed that VRE

was surviving in broiler houses despite cleaning procedures between production rotations (Heuer et al., 2002b).

C. Transfer from the environment to the clinic

Patients admitted to hospital are likely to acquire bacteria which are multiplying in the hospital environment, such as *Pseudomonas aeruginosa* and these may well be antimicrobial resistant. It has been demonstrated that the general environment of the hospital, particularly sites such as sink drains, mop heads and other wet environments will act as sources not only for bacteria capable of directly causing nosocomial infection but which can also act as a gene pool for antibiotic resistance genes. Outside of this obvious interaction of patients with the hospital environment, it is probably food that is the major route of flow of resistance genes from the more general environment to man. The human gut carries a large number of commensal bacteria, usually of the order of $\geq 10^{10}$/g of faeces and this figure only applies to culturable bacteria. The bowel flora is constantly being challenged by new bacteria in the food and although the dominant flora remains, colonisation with a minority of antibiotic resistant bacteria, particularly *Enterobacteriaceae*/enterococci and staphylococci occurs in those individuals not receiving antimicrobials. The size and complexity of this bowel flora reservoir which is in direct connection, it can be argued, with the environment, has long been recognised as containing large numbers of antibiotic resistant bacteria. A study undertaken as long ago as 1979, showed that in individuals with no history of recent consumption of antibiotics, 10% or more of the total aerobic Gram negative bacteria were resistant to one or more antimicrobials (Levy et al., 1988). This pool of resistant genes may transfer into bacteria with significant pathogenicity towards humans, or in very many cases, opportunistic

pathenogenic bacteria such as *E. coli, Klebsiella spp.*, entrococci and *Staphylococcus aureus* which may cause infections in the individual. In this way, there is a continuous link between selection for antibiotic resistance in the general and agricultural environment and human medicine. The other major commensal flora site on humans is the skin surface and recently with the recognition of community acquired MRSA, the importance of antibiotic resistant staphylococci and other Gram positive bacteria on the skin, and the transfer into potentially pathogenic species such as *Staphylococcus aureus* has been emphasised. As with the large bowel, all the studies clearly show that even those individuals not receiving antibiotics carry a substantial load of antibiotic resistant bacteria which must be strongly influenced by interactions with other humans, and companion and food animals. An extensive study by Cove and colleagues showed that the incidence of seven primary antibiotic resistance markers amongst the staphylococcal flora in antibiotic untreated subjects was tetracycline 87.5%, erythromycin 68.8%, fusidic acid 56.3%, trimethoprim 42.4%, chloramphenicol 25%, clindamycin 9.4% and gentamicin 4.7%. We now recognise that amongst staphylococci there is ample opportunity and genetic mechanisms for the mobilisation of those genes into potentially pathogenic species (Cove et al., 1990). Some special groups in the human community such as farm workers, medical personnel and patients receiving antimicrobials, have a much higher incidence of colonisation with antibiotic resistant bacteria, some of which, such as in the case of farm workers, are derived from contact with farm animals which have either been treated with antimicrobials or exposed to selecting agents. Recent problems with community acquired MRSA in pig farmers is a graphic illustration of the way in which problems can arise rapidly and undermine the use of clinically important antimicrobials.

V. MRSA in the non-clinical environment

A. Methicillin resistance in *Staphylococcus aureus*

Staphylococcus aureus is well known for its ability to acquire antibiotic resistance, both historically in relation to penicillin, erythromycin and tetracycline and more recently methicillin and vancomycin resistance. The acronym MRSA (Methicillin resistant *S. aureus*) is feared by health-care professionals the world over. *S. aureus* forms part of the normal human flora, residing asymptomatically in the mucosal linings of healthy individuals and at other moist skin sites (Hiramatsu et al., 2001; Peacock et al., 2001) and is particularly pathogenic in individuals at the extremes of age who have intravascular/urinary catheters, diabetes and other compromising co-existing medical conditions (Lindsay and Holden, 2004). However, recent years have seen a rise in highly virulent community acquired strains (CA-MRSA), capable of causing disease in young, healthy individuals with none of the prescribed risk factors. Resistance to methicillin is carried by SCC*mec*, a mobile genetic island that can disseminate horizontally, although its mode of transfer is currently unknown (Hanssen and Ericson Sollid, 2006). Resistance is encoded by the *mec*A gene (Ito et al., 1999), *mec*A encodes an attenuated penicillin binding protein (PBP2' or PBP2a) which has a lower affinity for penicillin and other β-lactams than the innate PBP's (Hartman and Tomasz, 1981), hence interfering with antimicrobial activity. There are 6 basic described SCC*mec* types; additional resistance genes may be present or absent depending on the type. SCC*mec* is inserted into the *S. aureus* chromosome near the origin of replication, always at the same location at the 3' end of the *orf*X gene (Kuroda et al., 2001). Its origins are unknown, but as no methicillin-susceptible *S. aureus* (MSSA) homologue exists (Archer and Neimeyer, 1994) it has been suggested that it was transferred horizontally from a coagulase-negative staphylococcus (CoNS)

such as *S. sciuri* (Couto et al., 1996; Couto et al., 2003). The conjugative transposon containing *tet*M (tetracycline resistance) has been suggested to pass between *Clostridia* and staphylococci (Ito et al., 2003), and empirical support for the movement of mobile elements between staphylococci and other low GC gram negative bacteria (Gill et al., 2005), and the potential transfer of *van*A (vancomycin resistance) from *Enterococcus faecalis* to *S. aureus* (Weigel et al., 2003) lends weight to the possibility of a common gene pool available to many bacterial species (Hanssen et al., 2004). Movement of DNA into *S. aureus* is tightly controlled by a restriction modification system Sau1, a type 1 system which has been found on the chromosome of all known sequenced strains (Waldron and Lindsay, 2006).

Although much of the genome of *S. aureus* consists of mobile genetic elements, because of the presence of Sau1, horizontal transfer is likely to be more frequent amongst other members of the same lineage, as the Sau1 restriction-modification system present in different lineages have specific differences (Waldron and Lindsay, 2006). This is thought to explain the rare occurrence of *vanA* carrying VRSA and the limited number of lineages with SCC*mec* (CC1, CC5, CC8, CC22, CC30 and CC45). Possession of SCC*mec* is thought to carry a fitness cost and will therefore only be selected for when strains have been exposed to antibiotics (Katayama et al., 2003).

B. Environmental reservoirs of MRSA

Although antibiotic selective pressure present in clinical environments is clearly a major source of MRSA infection (Dar et al., 2006), environmental reservoirs have been implicated in the spread of resistant strains. Sub-inhibitory levels of antibiotics may be to blame for inducing resistance in commensal bacteria in farm animals,

inducing resistance in pathogenic bacteria through plasmid transfer (Singer et al., 2003). In pig and poultry farming, heavy use of antibiotics in typically intensively farmed settings predisposes them to MRSA colonisation (Shea, 2004; van Den Bogaard et al., 2000). Close contact between animals is inevitable, and infections are likely to spread quickly. Whereas bacterial transmission in humans is (or should be) easily negated through regular hand washing, oral-faecal contact cannot be prevented in animals and fast transmission of faecal-borne disease is unavoidable (van den Bogaard and Stobberingh, 1999). Therefore, an infection in one or two animals is often combated by a blanket treatment with antibiotics of the entire house, which may contain over 10,000 animals (Shea, 2004). The farming practice employed may have some bearing on the antibiotic susceptibility of commensal *S. aureus*; organic farming may result in fewer resistant bacteria than in conventional farms, due to lower exposure to antibiotics and reduced contact between animals (Halbert et al., 2006; Sato et al., 2005; Tikofsky et al., 2003). There is some contrary evidence (Sato et al., 2004), but the continued use of antibiotics in some of the organic farms studied was a possibility (Busato et al., 2000).

Cross-resistance for methicillin and some cephalosporins has been demonstrated (Hansen-Nord et al., 1988; Menzies et al., 1987), with administration of cephalosporins increasing the acquisition of nosocomial MRSA three-fold (Asensio et al., 1996). Similarly, tetracycline resistance may be caused by one of several genes, one of which, *tet*K, is carried by a plasmid (pT181) that is inserted into SCC*mec*III (Ito et al., 2003). As tetracycline concentrations have been found to persist at high concentrations long after slurry application has ceased (De Liguoro et al., 2003; Hamscher et al., 2002), selection for tetracycline resistance may contribute to methicillin resistance as a result of selection for SCC*mec*III; although SCC*mec*IV is

far more common in community MRSA. In the absence of antibiotics, the presence of quaternary ammonium compounds (QACs) in soils, used extensively as disinfectants, may also co-select for β-lactam resistance (Sidhu et al., 2002; Sidhu et al., 2001); resistance to QACs correlates with the β-lactamase transposon Tn552 due to co-carriage of the *bla*Z gene and *qac*A (Anthonisen et al., 2002). Evidently, the introduction of any of these compounds to the environment may lead to selection for methicillin resistance in *S. aureus* colonising or infecting farm animals, or in other bacteria inhabiting the soil; there is some evidence to suggest colonisation of the rhizosphere by *S. aureus* may be possible, so acquisition of methicillin resistance may occur both in soil and in farm animals (Berg et al., 2005; Germida and Siciliano, 2001; Morales et al., 1996).

C. Pig associated MRSA

Antibiotic resistance levels vary from country to country. For example, the percentage of MRSA in the UK (40% of nosocomial *S. aureus* isolates causing bacteremia) contrasts sharply with that in Holland, where it is currently very rare, making up only 1% of *S. aureus* isolates (Tiemersma et al., 2004). In Holland most cases are found in people who have recently attended foreign medical institutions; however, occasional instances have arisen whereby foreign travel was not a contributing factor. One such case described an MRSA positive baby that had never travelled abroad (Voss et al., 2005). The family lived on a pig farm, and MRSA was isolated from one of the pigs. Two further cases were reported in the same study, both of which were associated in some way with pig farming. As pig farming was the only common denominator in these cases it was deduced that there may be a link between pig farming and increased risk of MRSA infection; indeed colonisation by MRSA in pig farmers was found to be

almost twice that of the general population (Aubry-Damon et al., 2004). Pigs have been similarly implicated as a reservoir of MRSA in France, a study of pig isolates found all to be non-typeable by pulsed-field gel electrophoresis (PFGE), the so-called "gold standard" for MRSA typing (Armand-Lefevre et al., 2005), and the association between PFGE non-typeable strains and pig farming has gained further empirical support (Huijsdens et al., 2006). That three multi-locus sequence types (MLSTs) ST9, ST398 and ST433 were found in pigs and only in humans associated with pig farming suggests an association between these STs and pigs (Armand-Lefevre et al., 2005). These findings were supported by a recent Dutch study in which 39% of all pigs sampled from slaughter houses (540 pigs) carried MRSA in their nares, and all of these belonged to ST398 (de Neeling et al., 2007). Obviously Holland represents an unusual case due to its low MRSA incidence, but the high percentage of swine carriers all of the same MLST type suggests a recent dissemination of MRSA. The vast majority of isolates contained SCC*mec* types IV and V, indicating a relatively frequent transfer of this mobile element; types IV and V are suspected to transfer at a greater rate than the other types (Daum et al., 2002). As pig-associated strains seem to be passed from human to human as well as between humans and pigs, pig farming may represent a source of CA-MRSA (Huijsdens et al., 2006). CA-MRSA is susceptible to a greater range of antibiotics than its hospital-acquired counterpart (Herold et al., 1998), but tends to be more virulent due to the acquisition of toxin genes such as the Panton-Valentine Leukocidin (PVL) (Boyle-Vavra and Daum, 2007). PVL has not yet been found in pig-associated strains (Huijsdens et al., 2006), but more studies need to be conducted to confirm this. There are relatively few CA-MRSA strains, with 6 currently recognised (Eady and Cove, 2003). It has been suggested that these strains are associated with particular niches (Shukla, 2005),

concurring with the potential association of the above sequence types with pigs and pig farmers. The antibiotic resistance patterns of pig MRSA, other than to methicillin, reflect the antibiotics used in veterinary medicine: tetracycline resistance is very common, whereas ciprofloxacin resistance has been found to be completely absent (de Neeling et al., 2007). This is very different to the situation in nosocomial MRSA strains, in which over 80% of MRSAs may be resistant to ciprofloxacin (Marangon et al., 2004; Raviglione et al., 1990), perhaps suggesting that ST398 is circulating in pigs and only infecting humans occasionally, or that pigs are an emerging reservoir of MRSA.

D. Cattle associated MRSA

MRSA has been considered comparatively rare in cows, and those *S. aureus* found to be resistant to methicillin tend to lack SCC*mec*, gaining resistance from the production of β-lactamases (De Oliveira et al., 2000). Penicillin and ampicillin resistance are more widespread (Gentilini et al., 2000; Guler et al., 2005) due to the frequent use of these antibiotics in treating intramammary infections (Pengov and Ceru, 2003). Antibacterial treatment for mastitis has been implicated as a major cause of resistance in bacterial isolates from treated animals, other animals within the herd, and in meat products intended for human consumption (Berghash et al., 1983; Chen et al., 2004; Griggs et al., 1994; Piddock, 1996). Furthermore, the secretion of antibiotics in milk produced by cows under treatment for mastitis is one of the most common causes of illegal antibacterial residues in milk (Erskine, 1996). Although cattle have a greater freedom of movement than intensively farmed pigs and poultry, effectively reducing the risk of transmission, other risk factors are present. There are extreme inconsistencies in the therapeutic success rates of the standard treatment regimes (Dingwell et al., 2003), with chronic infections being cured through intra-

mammary antibiotic infusions only 35% of the time (Owens et al., 1997). Indiscriminate use of antibiotics, incomplete treatments and incorrect diagnoses of antibiotic resistant strains do not help the situation (Turutoglu et al., 2006). Although methicillin is not generally used in treatment of cows, MRSA is increasing in its significance as a cause of bovine mastitis (Bernabé et al., 2005; Turutoglu et al., 2006). Acquisition of MRSA may occur through direct contact with humans: one outbreak of mastitis has been attributed to contact with a farm worker (Devriese et al., 1986), and more recent empirical evidence concurs with this (Fox et al., 1991; Roberson et al., 1994). However, this is slightly contentious as evidence to the contrary suggests that transmission between humans and cows is rare (Kapur et al., 1995; Lopes et al., 1990) due to the host-specificity of *S. aureus* clones (Smith et al., 2005).

Environmental survival of MRSA may contribute to bovine infections, as shown in the clinical setting as discussed by Hardy *et al*. (2004a), where it may survive for several months and act as a source for infecting patients (Neely and Maley, 2000; Sasatsu et al., 1993). Biofilm formation on various materials allows persistence and avoidance of desiccation (Gotz, 2002), so there is a risk of transmission from cow to cow via milking devices and other surfaces. Despite the move towards automated milking practices, reducing contact between humans and untreated milk, the production of aerosols in milking parlors may put workers, as well as other cows, at risk from contamination (Roberson et al., 1994). Similarly, aerial contamination has been noted in pig barns (Gibbs et al., 2004), and in aerosols produced by confined swine-feeding operations (Chapin et al., 2005; Sapkota et al., 2006), and pig feeds have been implicated as potential sources of contamination of tetracycline-resistant *S. aureus*, which may thrive in tetracycline enriched feeds (de Neeling et al., 2007).

E. Horse associated MRSA

MRSA has also been identified as an emerging disease in horses (Weese et al., 2005a; Weese et al., 2005b; Weese et al., 2006), which might act as a reservoir of rare strains for transmission to humans (Baptiste et al., 2005). The isolation of MRSA from two horses in Canada led to a larger study which revealed the colonisation of 79 hospitalised horses and also of 27 people involved in caring for these animals, strongly suggestive of frequent transmission between horses and humans (Weese et al., 2005b). All isolates in the study were from nasal swabs and belonged to the Canadian community associated strain C-MRSA5, which is uncommon in humans. Thus horses may act as a reservoir for this strain, perhaps mirroring the putative association of ST398 with pigs. In Slovenia, MRSA was absent from a total of 300 horses sampled with nasal swabs (Vengust et al., 2006); one explanation for this is that MRSA may also colonise the perineum or the throat, and this has been found to be the sole site of infection in some cases (Coello et al., 1994) so it is possible that sub-clinical carriers of MRSA were missed. Indeed, a study on humans in nursing homes indicated that up to 14% of cases are missed by screening the nares alone (Lee et al., 1997). MRSA colonisation tends to be transient in nature, and so may be missed on screening, whereas it has been shown that infections are likely to persist in hospitalised horses, particularly in those that have undergone invasive procedures (Seguin et al., 1999).

F. MRSA in companion animals

The situation in companion animals differs from that seen in pigs, as there does not appear to be any association with a particular MRSA clone, with the same strains found in humans (van Duijkeren et al., 2003), indicating that pets acquire the infection from humans. Although *S. intermedius* is the predominant staphylococcal species in

dogs (Biberstein et al., 1984; Hoekstra and Paulton, 2002), *S. aureus* is also frequently found on the skin or associated with suppurative infections (Kloos, 1990). Dogs may act as a reservoir of MRSA (Baptiste et al., 2005) and have been implicated in causing unexplained relapses in humans previously treated and cleared of MRSA infection (Manian, 2003; van Duijkeren et al., 2003). Strains isolated from humans and dogs have been found to have the same PFGE patterns (Manian, 2003) and SCC*mec* types (van Duijkeren et al., 2004). Following treatment of dogs, relapses of infection in the owners ceased (Cefai et al., 1994; Manian, 2003; van Duijkeren et al., 2004). Cats may also carry MRSA, although like dogs, *S. aureus* is not the most common staphylococcal infection, in cats this is fulfilled by a combination of *S. intermedius* and the CoNS *S. felis* (Lilenbaum et al., 1998; Patel et al., 1999). Surgery is a risk factor in cats and dogs just as it is in humans, making up the vast majority of MRSA cases (Boag et al., 2004; Owen et al., 2004; Tomlin et al., 1999; van Duijkeren et al., 2003).

The epidemiology of MRSA appears to be changing, with the possibility of animals acting as reservoirs of infection for people without any of the usual risk factors. In countries that currently have a low incidence of MRSA, this may be an important source of an otherwise rare infection.

VI. Conclusions

The evolution of antibiotic resistant bacteria is one of the most significant problems in modern medicine and posses a serious threat to human health. Increasingly the huge diversity of resistance genes which already exist in the environment is beginning to be appreciated. Understanding the selective pressures and mechanisms of gene transfer which drive dissemination of resistance genes, not only in the clinic, but also in the

wider environment is crucial for long term strategies in the treatment of microbial disease.

Modern farming practice is attempting to reduce dependency on antibiotics but this in itself may not reduce particular mechanisms of resistance such as genes carried on class 1 integrons, which we have shown can be selected for by biocides in the environment. Understanding the ecology of resistance genes is extremely difficult as genes may be carried by unculturable bacteria (99.0-99.9% of bacteria). Movement of genes between environmental bacteria and the clinic has therefore been difficult to investigate in the past. However, modern molecular approaches such as epidemiological studies of key resistance determinants in total community DNA using quantitative real-time PCR allows detailed analyses and comparison of gene prevalence in the environment and human gut. Functional metagenomics and integron clone library construction allows the entire resistance gene pool or metagenome to be analysed. Antibiotic resistant bacteria in the environment may be transferred to the human population via ingestion of contaminated food and water, via direct contact with animals, swimming in lakes, rivers and the sea and by airborne bacteria. In reality, there is no distinction between clinical and non-clinical environments; both must be considered to fully understand the underlying causes of antibiotic resistance in clinically important bacteria..

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