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# An investigation of the occurrence of antibiotic resistance genes among strains of *Staphylococcus aureus* isolated from animals and their relationship to human strains of *Staphylococcus aureus*.

**Andrew R. Robb B.Sc. F.I.B.M.S. C.Sci.**

**A thesis submitted for the Degree of Doctor of Philosophy at**
**The Open University, Department of Life Sciences**

Submission date: 21 April 2008
Date of award: 20 Oct 2008

## Dedication

**To Annette for her love and support and to the memory of my Mother Euphemia who would have been so proud.**

- 6 MAY 2009

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**Abstract**

Data has emerged which indicates that antimicrobial use in animals has created a reservoir of resistant bacteria and resistance genes that have spread to humans.

The aim of this study was to examine the influence of animal antimicrobial use on human strains of *S. aureus*. Phenotypic and genotypic methods assessed the genetic population structure, potential for host adaptation, frequency of antimicrobial resistance, presence and frequency of genes encoding tetracycline and macrolide resistance and structural variation in *tet*K genes in *S. aureus* from animals compared with human clinical strains. In addition, the transferability of *tet*K resistance plasmids from animal strains to *S. aureus* 8325-4 was investigated.

DNA based typing exhibited 100% typeability, high levels of discrimination and a high degree of concordance between methods. Multi-locus sequence typing (MLST) identified six sequence types (CC5, CC15, CC22, CC25, CC30 and CC45) common to both isolate collections that represented four of the five major human methicillin resistant *Staphylococcus aureus* (MRSA) clonal lineages. The relatedness of these clones was further supported by the analysis of 20 different virulence determinants.

Isolates of CC5 exhibited resistance to ciprofloxacin, clindamycin, erythromycin, penicillin, streptomycin, tobramycin and tylosin, CC15 to penicillin and tetracycline, CC22 to penicillin and rifampicin and CC30 to penicillin. Isolates of CC45 were fully susceptible. Low level biocide resistance was detected but the significance of this was unclear.

The *tet*K and *erm*C genes were the predominant tetracycline and macrolide resistance genes. *tet*K was harboured by animal isolates of CC5 and CC15 and *erm*C by animal isolates of CC5. Restriction fragment length polymorphism (RFLP) of *tet*K amplicons produced indistinguishable restriction patterns. High frequency transfer of a *tet*K plasmid from a chicken *S. aureus* (CC5) to *S. aureus* 8325-4 was observed.

These data support the hypothesis that animals represent an important reservoir of antibiotic resistant *S. aureus* with the ability for strain and antibiotic resistance gene transfer to humans.

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**Acknowledgements**

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**Final thanks to Mr Drummond McNair for giving me this opportunity and to all the staff at the Scottish MRSA Reference Laboratory for their assistance and support.**

## Publications and presentations

1. **Robb, A.R., D. Morrison, S. Dancer, C.G. Gemmell.** 2003. Antibiotic susceptibility and strain typing of animal Staphylococcus aureus. Clinical Microbiology and Infection 9; Suppl 1: 384.

2. **Robb, A., D. Morrison, S. Dancer, C.G. Gemmell.** 2004. Expression of resistance of tetracycline in staphylococcal strains isolated from humans. Proceeding & Abstracts of the XIth International Symposium on Staphylococci and Staphylococcal Infection, Charlston, South Carolina, USA.

3. **Roberts, S., K. O'Shea, D. Morris, A. Robb, D. Morrison, S. Rankin.** 2005. A real-time PCR assay to detect the Panton Valentine leukocidin toxin in staphylococci screening *Staphylococcus schleiferi* subspecies *coagulans* strains in companion animals. Veterinary Microbiology 25;107(1-2):139-144.

4. **Robb, A., D. Morrison, S. Dancer, C.G. Gemmell.** 2005. Expression of tetracycline resistance in staphylococcal strains isolated from animals. Proceeding & Abstracts for Antibiotic use in agriculture:the road to prudent use Toronto, Canada.

**Declaration**

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## Chapter 1 Introduction

### 1.1 General Introduction

"Antibiotic resistance as a phenomenon is, in itself not surprising nor is it new. It is however, newly worrying because it is accumulating and accelerating, while the world's tools for combating it decreases in power and number" (Joshua Lederberg: Nobel prize winner, 1958)

Almost 50 years later the levels of antimicrobial resistance has reached an alarming level with the real potential to enter a post antibiotic era[259]. Worldwide there is serious concern over the soaring levels of antibiotic resistance detected in a wide variety of clinical and environmental organisms[61,125]. When a growing portion of bacteria develops resistance, effectiveness of our current treatments and our ability to control many infectious diseases will be jeopardized. It is generally accepted that the main risk factor for the increased resistance seen in pathogenic bacteria is the increased use of antibiotics which has led to the emergence and dissemination of resistant bacteria and resistance genes[449].

Some reports have suggested that most of the antibiotic resistance problems in humans stem from overuse or from inadequate controls in human medicine. While the consumption and the rates of antibiotic resistance in humans has definitely increased within Europe, especially the southern and eastern states there is an increasing interest in the range of antibacterial compounds being used for clinical and nonclinical purposes especially in the fields of animal husbandry, horticulture and fish farming and the risk of transmission of antibiotic resistance from animals to humans [291,161].

## 1.2 Antibiotic use in agriculture

One of the central issues in this area is the fact that some antibiotics commonly used in animals both for therapy and growth enhancement are either identical to or are related to agents used in humans (Table 1.1) with relatively few animal specific agents such as streptothricin and coccidiostats[126,335,352,376,222,475, 147,232,45,46,363,462,326,338,482,162,385].

Table 1.1 Antibiotics commonly used in agriculture

| Antibiotic class | Animal | Human |
|---|---|---|
| Aminoglycosides | Avilmycin, Apramycin, Spiramycin, **Gentamicin** | **Gentamicin**, Kanamycin, Tobramycin, Amikacin |
| Amphenicols | **Chloramphenicol** | **Chloramphenicol** |
| Cephalosporins | Cephadroxil, **Cefuroxime**, Cephalexin | Ceftriaxone, Cefotaxime, **Cefuroxime** |
| Glycopeptides | **Avoparcin** | Vancomycin, Teicoplanin |
| Lincosamides | Lincomycin | Clindamycin |
| Macrolides | Tylosin | Erythromycin |
| Nitrofurans | **Nitrofurantoin** | **Nitrofurantoin** |
| Penicillins | **Penicillin** | **Penicillin** |
| Fluroquinolones | Enrofoxacin | Ciprofloxacin |
| Pleuromutilins | Tiamulin, **Valnemulin** | **Valnemulin** |
| Polyenes | **Amphotericin B** | **Amphotericin B** |
| Polypeptides | **Bacitracin** | **Bacitracin** |
| Streptogramins | Virginiamycin | Synercid |
| Tetracyclines | **Tetracycline, Oxytetracycline, Doxycycline, Chlortetracycline** | **Tetracycline, Oxytetracycline, Doxycycline, Chlortetracycline** |
| Sulphonamides | Sulfadoxine, Sulfatroxazole, Sulfadiamine | Sulphamethoxazole |
| Others | Framycetin, **Fusidic acid, Polymixin B**, Tiamulin, Tilmicosin, Nourseothricin | Isoniazid, Rifampicin, Ethambutol, **Polymyxin B, Fusidic acid** |

Antibiotics have been used in clinical medicine for over 60 years and were introduced into veterinary practice soon after[490].

There are four ways in which substances exhibiting antimicrobial activity are used in animals; therapy[336], metaphylaxis[385], prophylaxis[493] and growth promotion[232].

### 1.2.1 Therapeutic use of antibiotics

Therapeutic use of antibiotics is intended to treat and control existing bacterial infection and involves using curative doses of antibiotic agents for a relatively short period of time[391]. The modes of application of antibiotic agents for therapeutic purposes differ with respect to the size of the group of animals. In principle, individual animal treatment has to be differentiated from group treatment. Individual animal treatment is usually offered for domestic pets[168], as well as horses used for sport and leisure. Among food-producing animals, individual antibiotic therapy is most commonly used in the treatment of mastitis in dairy cattle and respiratory infection in calves[51]. Individual antibiotic therapy is comparable to the use of antibiotics in human medicine where every diseased animal is examined which may include laboratory analysis, such as identification of the pathogen and antibiotic susceptibility testing, and antibiotics only given to animals showing signs of clinical disease, either orally or perenterally, and the dose given being appropriate to the animal and particular pathogen[385].

The nature of modern animal production systems, particularly pig and poultry, make individual treatment impractical and for fish production mass medication is the only feasible means of treatment[2,164,373]. Many antibiotics are administered in water or feed to animals raised in large numbers under industrial conditions e.g. 10,000-30,000

chickens/turkeys in a flock, 100 weaner pigs in one pen[385] or >30,000 beef cattle in a single herd[289].

### 1.2.2 Prophylactic use of antibiotics

Prophylactic or strategic use of antibiotics, at therapeutic levels, are used when the spread of clinical disease cannot be contained by vaccination, changes in animal management, or improvements in hygiene and when the development of disease in animals in contact with an infected source is virtually inevitable without antibiotic intervention [208,290]. Numerous modern animal production systems rely on the prophylactic use of antibiotics at particular periods in the production process. For instance, in dairy cattle, the intramammary administration of antibiotics at the end of the lactation period has been shown to prevent mastitis[51]. In addition, the production of pork and beef both rely on the prophylactic use of antibiotics during weaning or mixing of animals from different herds. It has been shown that without this intervention an increase in respiratory and enteric infection can occur severely compromising animal welfare[385].

### 1.2.3 Metaphylactic use of antibiotics

Metaphylaxis is a specific form of disease prevention in which all animals in a herd or flock are treated when a set proportion of animals become diseased during a defined period, and the probability of most or all of them getting infected are high[208]. Early medication of the entire animal group has been shown to improve morbidity and mortality rates while reducing the amount of antibiotics required in the treatment of a large clinically ill population.

### 1.2.4 Antibiotics used for growth promotion

Antibiotic growth promoters (AGP) are antimicrobial drugs added to animal feed at subtherapeutic levels to enhance growth, improve feed efficiency[45,127,221,426] and reduce waste production in food animals[449]. In the past, it was recognised that subtherapeutic levels of antibiotics had a positive effect on growth performance and herd health. Feeding antibiotics to animals has been shown to significantly increase weight gain[226] and to improve feed utilization by up to 7%[453]. Antibiotics used for this purpose have also been called performance enhancers, feed savers and antimicrobial feed additives[482]. The mechanisms of growth promotion are still not exactly known[54]. Experiments with germ-free chickens have indicated that the action of the AGP is mediated by their antibacterial effect[131]. Four hypotheses have been proposed to explain their action: (i) nutrients may be protected against bacterial destruction; (ii) improved digestion, metabolism and absorption of essential nutrients such as carbohydrates, proteins, amino acids, minerals and vitamins may improve because of thinning of the small intestinal barrier; (iii) the antibiotics may decrease the production of toxins by intestinal bacteria; and (iv) there may be a reduction in the incidence of subclinical intestinal infections[54,131]. It has also been reported that the use of AGP may have additional benefits for the environment, with reductions in the excretion of methane, nitrogen and phosphorous and reduced fly survival. And for the animal due to better heat tolerance and improved immune status[247]. The use of antibiotics as AGP has been a hallmark of modern intensive farming practice, but as detailed below their use is not without criticism[162,447,470].

While the distinction between the four uses of antibiotics in animals seems clear the terminologies are not uniform. The American Veterinary Medical Association (AVMA) defines "therapeutic" as including treatment, control and prevention of bacterial disease. Also the distinction between prophylaxis and growth promotion is less clearly defined than between prophylaxis and therapy. In the United States of America (USA) some antibiotics may be approved for both purposes, and some growth promoters may help to prevent disease, even at nontherapeutic doses. This is an important point as the administration of antibiotics for limited periods can almost always be justified on the grounds of disease prevention and animal welfare[289].

### 1.3 Antibiotic consumption in agriculture

There is significant concern over the amounts of antibiotic used in agriculture. Accurate figures on the global antibiotic use/consumption in animals are hard to obtain[447]. However, it has been estimated that approximately $1x10^6$ t of antibiotics have been used in the last 50 years with $5x10^5$ t used in the treatment of disease in animals, soft fruits and vegetables[376], fish[164,334,373], for plant propagation in horticulture[128] and also as AGP[54,64,75,127]. Most data on antibiotic use in animals are based on the monetary value of sales of antibiotics, which distorts the data, with published figures estimating that the use of antibiotics in humans can be up to 20 times higher than in animals[76]. Antibiotics used in human medicine are more expensive per gram of active compound than those used in veterinary medicine and are significantly more expensive than those used as AGP[74]. It has been reported that this method of estimating the consumption of antibiotics in animals can be very misleading[447].

### 1.3.1 Agricultural antibiotic consumption in Europe

The availability of data on the consumption of antibiotics in agriculture in Europe is limited. A report published in 1995 estimated the world animal antibiotic market to be worth 11 billion Euros and that the European Union (EU) accounted for 30% of this market with 48% therapeutic use and 37% used as feed additive[248].

Across Europe there are large differences in the use of antibiotics in animal production between different countries. Prior to the 1999 EU restriction on the use of AGP the consumption of antibiotics in Belgium, France, Ireland, and the Netherlands, countries with large intensive animal production systems, for both growth promotion and therapy were thought to be equal. In contrast, the use of AGP in Austria has been reported to be three times higher than the therapeutic use of antibiotics[447]. However, in Sweden and Finland, countries that have historically been more restrictive in the use of AGP, <1% of all antibiotics used in animals were given for this purpose[242]. Conversely, the use of antibiotics for growth promotion in Germany, Greece, Italy, Portugal, Spain, and the UK are estimated to be 15%-50% lower than those used therapeutically[447].

Limited information is available regarding the consumption of different antimicrobial agents in different countries or for different animal species. Studies have reported the approximate consumption of antibiotics, in animals, across Europe for specific years[8]. Helmuth *et al* report that in 1984 1,520 t of antimicrobials were used in animals both therapeutically and as growth promoters within Europe[180]. Data from 1997 determined that antibiotic use in animals had increased by 335% to 5,093 t, of which 3,494 t (68.6%) were being used therapeutically, with tetracyclines accounting for 2284 t (65%)[426] and 1210 t of antibiotics frequently used in human clinical medicine including

macrolides, penicillins, aminoglycosides, co-trimoxazole, fluoroquinolones, cephalosporins, amphenicols, lincosamides, polypeptides, nitrofurans, steroid antibiotics, polyenes and pleuromutilins (Table 1.1). The remaining 1,599 t (31.4%) of antibiotics were given to livestock for growth improvement[385].

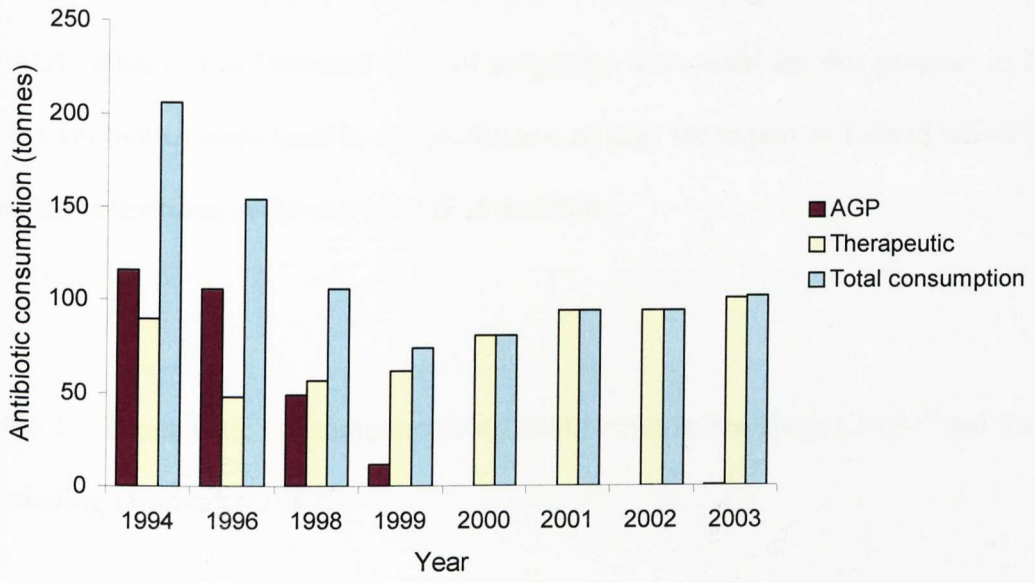
Based on body mass the mean therapeutic antibiotic dose has been estimated to be 241 mg/kg for humans and 54 mg/kg for animals[203]. However, there are large differences in the doses of antibiotics given to animals in countries across Europe. This is thought to be related to the intensity of farm production. In Austria, Ireland, Denmark, Finland and Sweden the therapeutic antibiotic dose given to animals are well below the mean at 6mg/kg, 12 mg/kg and 24 mg/kg respectively. In contrast, within countries such as Spain, Greece and the United Kingdom the therapeutic dose given is 103 mg/kg, 134 mg/kg and 148 mg/kg, respectively. These levels far exceed the mean therapeutic dose and suggests the imprudent use of antibiotics in these EU states[426].

Data on the overall use of antibiotic formulations have been published sporadically from countries within Europe and Scandinavia but generally do not include specific figures on antibiotic use in different animal species or groups of animals nor are there any figures on rates of resistance among bacteria from animals[168,289].

Denmark is different and since 1994 has monitored and published trends in, (i) the consumption of antimicrobial agents (Figure 1.1) (ii) the association between consumption and the occurrence of resistance, (iii) the resistance among bacteria from

animals, food and humans, and (iv) models transmission of resistance from animals to humans [3,26,336].

Figure 1.1 Antibiotic consumption in animals in Denmark (t)




During the period 1996-1999 Denmark terminated the use of AGP which lead to a 50% reduction in their total consumption of antimicrobials. This also resulted in a 76% increase in the therapeutic use of antimicrobials from 1998 to 2003. Even taking into account the significant increase in the therapeutic use of antibiotics the overall consumption of antimicrobials in 2003 is less that half the 1994 figure[3].

Data from 2003 indicate that tetracyclines were the predominant antibiotic group used in food animals accounting for 26.6% of all antibiotic use. This was significantly lower than tetracycline use in Europe. In contrast, other antibiotics (β-lactamase susceptible penicillins, aminoglycosides, cephalosporins, sulphonamide/trimethoprim, sulphonamides, macrolides, lincosamides and tiamulin) were used at a higher level in Denmark (Table 1.2). Approximately 80% of the Danish veterinary antibiotic consumption is used in the production of pigs. It is interesting to note that while the use of AGP is banned in Denmark 0.4 t of antibiotics were used for this purpose in 2003. These antibiotics were used in the production of pigs for export to Poland where there were no restrictions in the use of AGP at that time[3].

Table 1.2 Therapeutic consumption of antibiotics (%) in Denmark (2003)[3] and Europe (excluding Denmark) (1997)

| Antibiotic groups | Denmark | Europe |
|---|---|---|
| Tetracyclines | 26.6 | 65 |
| Penicillins | 18.5 | 9.5 |
| Aminoglycosides | 11.4 | 4.4 |
| Cephalosporins | 10.8 | N/A |
| Trimethoprim/ Sulphamethoxazole | 10.3 | 2.1 |
| Sulphonamides | 0.8 | N/A |
| Macrolides/Lincosamides /Tiamulin | 20.1 | 12.1 |
| Other undifferentiated agents | 1.4 | 6.6 |

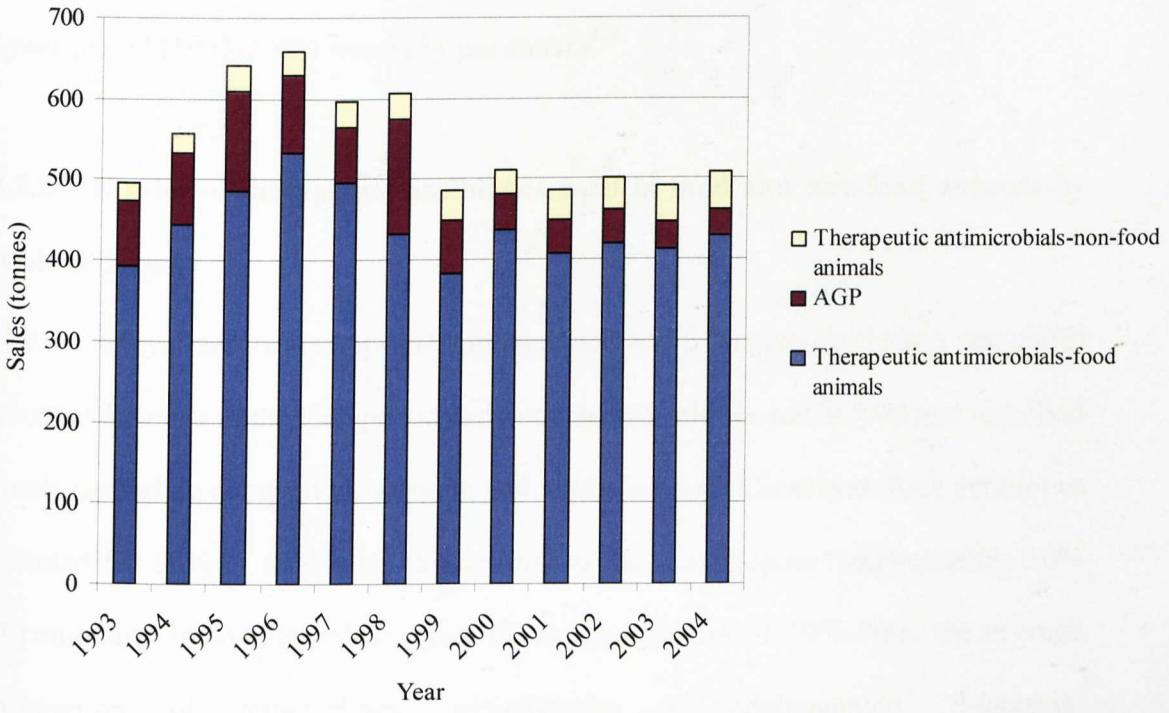
N/A, data not available

### 1.3.2 Antimicrobial sales figures for food and non-food animals in the UK for the period 1993-2004

#### 1.3.2.1 Total consumption

The Veterinary Medicines Directorate (VDM) is responsible for the authorisation of veterinary medicines in UK and in February 2001 and 2005 published sales figures for antibiotics used as veterinary medicine products and growth promoters for the period 1993-2004 (Figure 1.2)[428].

Figure 1.2 Sales of antimicrobial therapeutic products and growth promoters (t) for the period 1993-2004 in food and non-food animals in the UK[428].



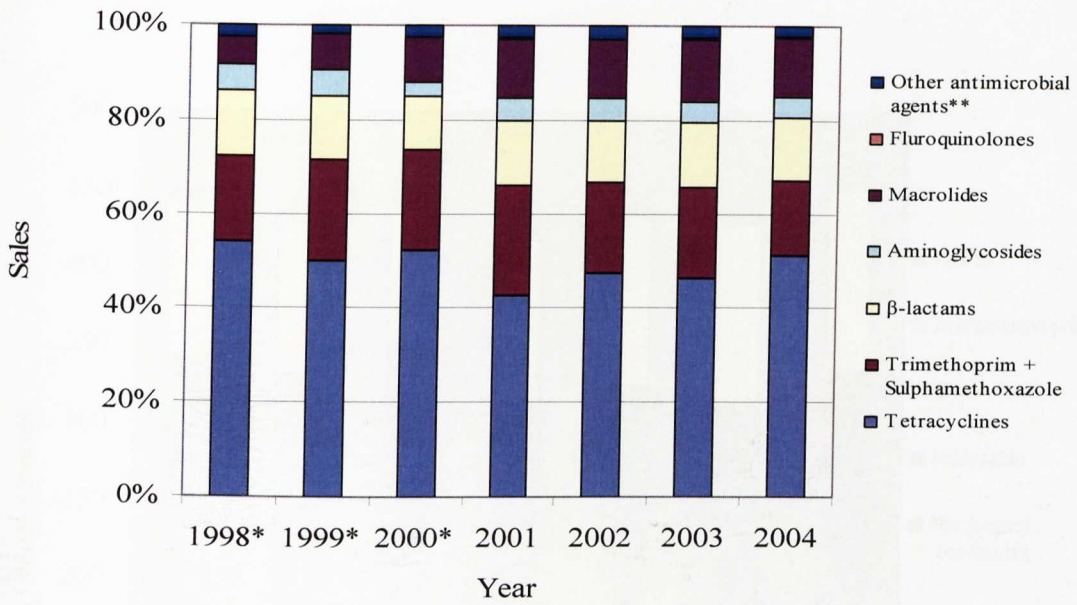
Between 1993 and 1996 the total sales of antimicrobial therapeutic products and growth promoters used in animals increased by 33% (495 t to 659 t) then declined steadily to their lowest level in 1999 when legislation was passed by the EU restricting the use of bacitracin, spiramycin, tylosin and virginiamycin as AGP[430]. There was a 5% increase in overall sales in 2000 due to the higher therapeutic use of tetracyclines, trimethoprim-sulphonamide and macrolides in pigs, poultry and multi-species products. This increased therapeutic use of antibiotics was thought to be due to the EU restrictions on the uses of antibiotics as AGP and the increased presence of diseases such as Porcine Dermatitis and Nephritis Syndrome and Post-Weaning Multi-System Wasting Syndrome (PDNS/PMWS) in pig production[428]. However, the total sales figures for antimicrobial therapeutic products and growth promoters over the period 1999-2004 remained broadly stable with sales in 2004 only 2.6% higher than those for 1993. Nevertheless, the average overall sales of therapeutic antimicrobials and AGP over this 12 year period (1993-2004) was 545 t per annum[428].

### 1.3.2.2 UK sales of therapeutic antibiotics used in food and non-food animals by antibiotic group

In 2004 tetracyclines, trimethoprim+sulphonamide and β-lactams (including penicillin) accounted for most of the therapeutic antibiotic agents sold for use in food and non-food animals (including companion, working and wild animals). Combined these antibiotics accounted for 89% of total sales (tetracycline 63%, trimethoprim+sulphonamide 20% and penicillins 16%) (Figure1.3). Over the seven year period 1998-2004 the average consumption of tetracyclines, trimethoprim + sulphonamide, β-lactams, aminoglycosides and macrolides was 216 t, 88 t, 58 t, 18 t and 46 t, respectively. There has been a decreasing trend in the sales of trimethoprim + sulphonamide and an

increasing trend in the sales of macrolides whilst sales have remained relatively stable for the classes β- lactams, aminoglycosides, fluoroquinolones and other antimicrobial agents (Figure 1.3)[428].

Figure 1.3 Sales of antimicrobial therapeutic agents (%) in food and non-food animals for the period 1998-2004[428].




* Figures for sales of antimicrobial therapeutic agents in food animals only.

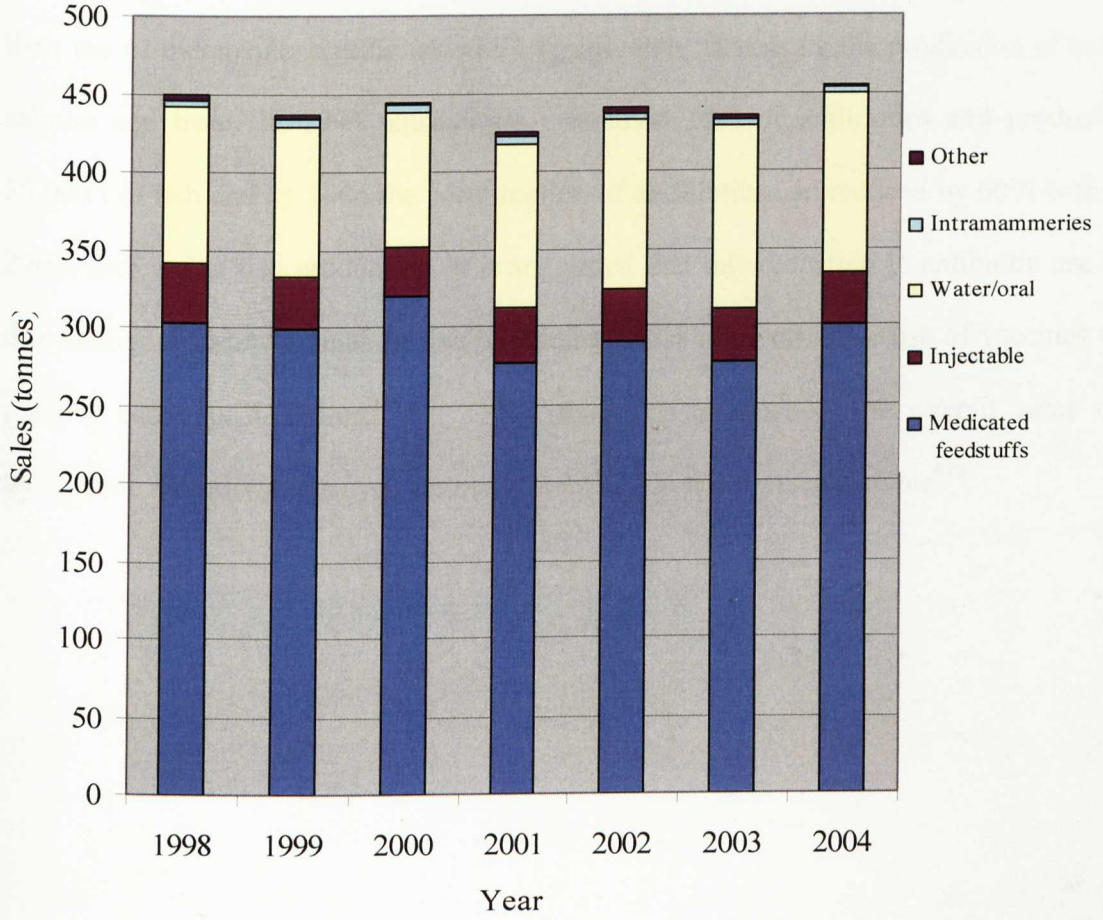
** These include Lincomycin, tiamulin, valnemulin, florfenicol, Novobiocin, decoquinate and diclazuril.

### 1.3.2.3 Antibiotic formulations and routes of administration.

Prior to the restrictions on the use of AGP 90% of all antibiotics used in food animal production were administered via medicated feed or by a water/oral route, which is an indicator of AGP use. In 2000, one year after the EU ban on AGP, this figure was 92% and remained constant at 90-91% through 2001-2004 (Figure 1.4)[428].

Therefore, unlike Denmark, the restriction on the use of antibiotics as growth promoters across the EU did nothing to reduce the overall sales of antibiotics, the antibiotic groups used or the methods used to administer these agents.

Figure 1.4 Sales of antimicrobial therapeutic agents (t active agent) by route of administration in food animals for the period 1998-2004



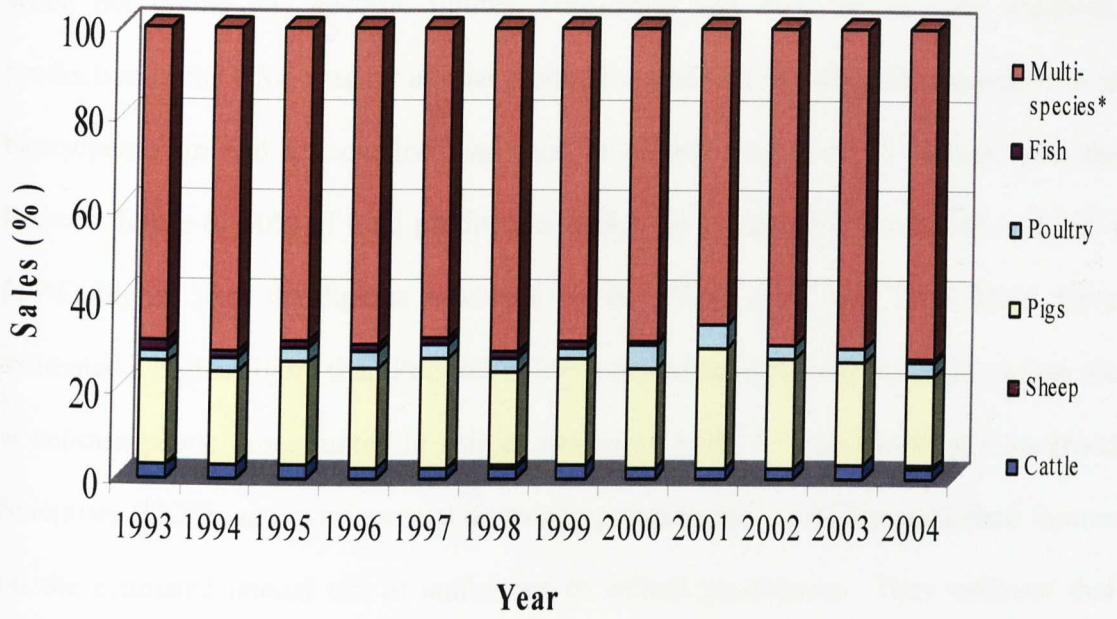
### 1.3.2.4 Sales of therapeutic antimicrobial agents by food animal species in the UK

While the VMD has attempted to give clear and accurate data on antibiotic consumption among a range of food animals they have found it impossible to determine the exact usage in different species.

It is clear from the data that approximately 70% of antibiotic products are approved for use in more than one animal species[428] (Figure 1.5).

Over the 12 year period (1993-2004) aquaculture has made a significant reduction in their use of therapeutic antibiotics while significantly increasing the production of both salmon and trout. In 1993 aquaculture consumed 10 t of antibiotics and produced 55,000 t of fish and by 2004 the consumption of antibiotics had reduced by 60% with a 290% increase in fish production. It is suggested that this reduction in antibiotic use is due mainly to improvements in fish husbandry and a more effective use of vaccines to prevent bacterial infections[164,373]. Other than fish production the overall sales of therapeutic antimicrobials over the period 1993-2004 has remained stable[428].

Figure 1.5 Sales of therapeutic antimicrobial agents (%) by food animal species 1993-2004.




* A combination of two or more of the following – cattle, pigs, sheep and poultry

### 1.3.3 Agricultural consumption of antibiotics in the USA

Many of the antibiotics commonly used in animals both for therapy and growth enhancement within the USA are either identical to or are related to agents used in humans including penicillins, tetracyclines, cephalosporins, fluoroquinolones, glycopeptides, aminoglycosides and streptogramins (Table 1.3)[134,226].

Reliable data on the use of antibiotics in animal production are not publicly available, making it difficult to determine which drugs are used in what quantities and for what purposes[289]. Within the USA no single government agency is able to supply accurate data on the amounts of antibiotic used in animals. Estimates of antibiotic consumption have been produced based on figures compiled from a variety of agencies including the Food and Drug Administration (FDA), Centre for Disease Control (CDC) and United States Department of Agriculture (USDA). Figures published by these agencies in 2001

indicate that 16,000 t of antibiotics are used in agriculture each year and that 70% (11,200 t) are used as AGP in the production of pigs, cattle and poultry[142]. Prescott[342], while not giving any specific figures, considered that 40% of the total antibiotic production in the USA was for animal production and that 55-60% of the production of benzylpenicillin and tetracycline was used in animal feed. Levy[261] agreed with the Prescott figure of 40% of total production, which he estimated to be 23,000 t, 7,000 t (44%) higher than the figures produced by the FDA, CDC and the USDA. Levy estimated a higher figure than Prescott's (80%) for tetracycline and benzylpenicillin use at subtherapeutic doses mixed in animal rations as AGP[259]. The Union of Concerned Scientists (UCS), an environmental advocacy group in the USA, has published figures on the estimated annual use of antibiotics in animal production. They estimate that 12,100 t of antibiotics are used each year with 11,200 t (92.6%) given to animals for non-therapeutic purposes while only 900 t were given in treatment of disease[162]. In contrast, a survey by the Animal Health Institute, a lobby group for the meat and livestock industry, reported in 2001 that only 8,100 t of antibiotics were used in animals with 6,700 t (83%) used therapeutically, either treatment or prophylaxis, with the remaining 1,400 t (17%) used as AGP[142].

Groups supporting the use of AGP indicate that their use protect animals from a variety of infectious diseases[64]. While animal welfare groups disagree with these claims and suggest that the long term use of antibiotic prophylaxis is unnecessary[489].

The overuse of antibiotics, whether in human medicine or agriculture, is a serious problem[260]. The UCS suggests that the agricultural use of antibiotics is likely to be a larger part of the overall antibiotic resistance problem than is currently thought and

conclude that it is a general misconception that human medicine is the major abuser of antibiotics and that it is the production of food animals where the abuse is occurring[142].

Table 1.3 Examples of antimicrobials approved for use in the United States of America in food animals.

| Purpose | Cattle | Pigs | Poultry | Fish |
|---|---|---|---|---|
| Treatment | Amoxicillin* | Amoxicillin* | Erythromycin* | Ormetoprim |
| | Cephapirin* | Ampicillin* | Fluoroquinolone* | Sulphonamide* |
| | Erythromycin* | Chlortetracycline* | Gentamicin* | Oxytetracycline* |
| | Fluoroquinolone* | Gentamicin* | Neomycin* | |
| | Gentamicin* | Lincomycin* | Penicillin* | |
| | Novobiocin | Sulfamethazine | Spectinomycin* | |
| | Penicillin* | Tiamulin | Tetracycline* | |
| | Sulphonamides* | Tylosin | Tylosin | |
| | Tilmicosin | | Virginiamycin | |
| | Tylosin | | | |
| Growth promotion and feed efficiency | Bacitracin* | Asanilic acid | Bambermycin | |
| | Chlortetracycline* | Bacitracin* | Bacitracin* | |
| | Lasalocid | Bambermycin | Chlortetracycline* | |
| | Monensin | Chlortetracycline* | Penicillin* | |
| | Oxytetracycline* | Erythromycin* | Tylosin | |
| | | Penicillin* | Virginiamycin | |
| | | Tiamulin | | |
| | | Tylosin | | |
| | | Virginiamycin | | |

* Antimicrobial agents used in human medicine

### 1.4 EU ban on AGP

The era of antibiotics as growth promoters (AGP) began in 1946 with the recognition of a substantial growth response to the inclusion of streptomycin in the feed of chickens[54]. By 1949 it was shown that both pigs and chickens fed a diet supplemented with the dried biomass recovered from the fermentation of *Sterptomyces aureofaciens*, used in the production of chlortetracycline, had a significantly improved daily weight gain ranging from 3 to 19%[54,116,226]. Antibiotics were approved for use as AGP in the USA in 1949 and by 1953 UK approval was also given[490]. At a time when livestock management was changing rapidly from low-performance, high mortality, free range farming to a more controlled and intensive husbandry, and when post war demands on increased food production were high, the discovery of an unexpected method to accelerate animal growth was received with interest and enthusiasm by scientists, agriculture and the general public. In April 1950 the discovery of AGP was front-page news in both New York and London[134]. However, the down side of this was that by the mid-1960's transferable antibiotic resistance was detected in *Salmonella* sp from farm animals[134]. Such was the concern that the UK government instructed a select committee, chaired by Michael Swann, to investigate the possible human clinical consequences of the use of antibiotics as AGP.

The Swann report was published in 1969 and concluded that molecules that are used for therapy in man and/or animals may not be used as an agricultural growth promoter[420]. Legislation restricting the use of antibiotics of therapeutic value in human medicine, particularly as feed additives, was introduced in 1971[493]. As a result, no antibiotics of human clinical importance are used for growth promotion in agriculture in the U.K., although closely related antibiotics were not restricted by legislation, and a veterinarian

must prescribe all antibiotics given to animals either therapeutic or prophylactic[493]. The USA has consistently refused to follow the recommendations of the Swann report using the argument of economic saving in favour of the use of antibiotics to promote growth, increase feed efficiency and to decrease waste production[29,296]. Since 1977, however, the Food and Drug Administration (FDA) has called for greater restriction in the use of both penicillin and tetracycline alone or in combination in animal feed. The US congress has opposed the FDA proposal basing their argument on the lack of evidence that antibiotic resistant bacteria of animal origin were frequently transmitted to humans although it was accepted that antibiotic use in animals may select for resistant bacteria[247].

Following the Swedish governments publication of the feedstuffs Act in January 1986 Sweden introduced a restriction on the use of antibiotics in animal feed, excluding the treatment, prevention or cure of diseases, meaning there use for growth promotion should not be allowed[449]. In March 1995 the first reports on the occurrence of avoparcin and vancomycin resistance in enterococci (VRE) in pigs and poultry were published[9]. At this time Danish farming organisations agreed a voluntary ban on the use of avoparcin. This was followed by a ban imposed by the Danish government and was also reported to the European commission under directive 70/524/EEC that allows member states, as a result of new information, to temporarily suspend the use of an approved feed additive if it constitutes a danger to animal or human health. The Danish ban was based on; (1) cross resistance between avoparcin and vancomycin, a very important antibiotic in human medicine, (2) the resistance was transferable, and (3) the use of avoparcin as an AGP selects for VRE and these VRE can transfer to humans via the food chain. Following the Danish decision to ban avoparcin Norway and Germany also

banned its use in June 1995 and January 1996 respectively[445]. However, in May 1996 the European Union scientific committee on animal nutrition (SCAN) concluded that there were insufficient data to support the ban of avoparcin and that further evidence was required to establish the risk to human health, animal health or the environment. Nevertheless, SCAN did accept that serious questions concerning the safety of avoparcin had been raised and stated that its use should be reconsidered. Following this the EU proposed that in a climate of uncertainty and to avoid any risk a temporary ban should be placed on avoparcin as an AGP in all EU member states which came into force in April 1997 under directive 97/6/EC[242]

On the 16th January 1998 Denmark banned the use of virginiamycin as an AGP due to the risk on the selection of streptogramin resistance in enterococci in pigs and poultry. This step was taken to protect the lifespan of synercid, a recently licensed streptogramin used to treat multidrug resistant infections in humans[11,45]. Pfizer, the producers of virginiamycin, challenged this decision before the European court of justice but lost the law suit when the court ruled that the ban on the product was not a disproportionate measure given the need to protect public health. In December 1998 the agriculture ministers of the EU member states voted in favour of the proposal to ban a further four AGP. The use of virginiamycin, bacitracin zinc, tylosin phosphate and Spiramycin as AGP were restricted, under directives 2788/98/EC and 2821/98/EC, from July 1999 allowing only the use of avilamycin, bamberimycin, salinomycin and monensin[242,430]. As of January 2006 the use of all remaining AGP has been restricted.

## 1.5 The debate

It is generally accepted that animals and humans constitute overlapping reservoirs of resistance, and consequently the use of antibiotics in animals can impact on public health[472]. Nothing in the area of antimicrobial use has generated a more vigorous debate recently than the use of antimicrobials in animals, especially food animals[436]. Fundamentally two positions have emerged. The first states that resistance to antibiotics of human importance has been generated in animals, is spread to humans with the potential to cause major harm, and therefore action must be taken to minimize it. This is the position adopted by regulatory officials in the European Union, Australia, Japan, New Zealand, and other nations and is designated the PRECAUTIONARY PRINCIPLE[31]. The second position states that resistance to antibiotics of human importance has been generated in animals, but the evidence that it has spread to humans, caused infection and treatment failure of these infections is minimal or non-existent, and that the ban on the use of antibiotics as growth promoters is unwarranted and should be revoked[336]. This position is known as the PRINCIPLE OF PROOF, which requires the gathering of evidence that a problem has emerged before taking action, and has been adopted by the USA. Although within the USA there is still much debate over this issue. The Food and drug administration (FDA) have recommended that the use of penicillins and tetracyclines as AGP should be severely restricted but Congress has overruled the FDA and after a review of these recommendations the National Academy of Science (NAS) concluded that no restrictive action should be taken on the use of AGP in animals.[134]

While both positions agree that resistance to antibiotics of human importance has been generated in some food animals evidence of harm to humans has been harder to find. Simply showing that a growing proportion of commensal and pathogenic bacteria isolated from food animals are resistant to antibiotics is not enough to prove a threat to human health[30].

Casewell *et al* report that following the ban of AGP in Sweden there were signs of adverse consequences for animal health and welfare resulting in economic consequences for farmers. They also report that in Denmark since the late 1990's poultry producers have been struggling with leg and skin problems among chickens and indicate that these problems compromise animal welfare[64]. In contrast, Weirup has reported that following the ban on AGP in Sweden there were no negative clinical effects reported in slaughter pigs, cattle and turkey. However, he did note that there had been an increased incidence of necrotic enteritis in chickens and diarrhoeal problems in weaning piglets that was quickly resolved and concluded that under good production conditions it is possible to reach good and competitive production without the use of AGP[481]. Emborg has published data on poultry production, in Denmark, after the ban on AGP and concluded that despite the fear of decreased productivity and increased mortality it was possible to withdraw all use of AGP, from broiler production, without a loss in output[116].

While the ban on AGP and the effect on animal welfare remains unresolved what is clear is that there has been a marked increase in the therapeutic use of antibiotics in the UK[428] and throughout Europe[64].

### 1.5.1 The evidence for the spread of antibiotic resistance from animal to human

Much of the evidence relating to the potential for transfer of a resistance problem from animals to man comes from a consideration of the epidemiology of zoonoses, mainly salmonella and campylobacter infection, and what have become known as indicator organisms, enterococci and *E. coli*, which cause opportunistic infections in animals and can cause disease in man which might be zoonotic[58,72,87].

The important antibiotic resistant strains in this context are multiply antibiotic-resistant salmonella[139], macrolide and quinolone-resistant campylobacter[121,199], glycopeptide and streptogramin-resistant enterococci[184,206,432], and multiply antibiotic-resistant *E. coli*[284,348]

The mechanisms of spread of antibiotic resistance from food animals to humans remains debatable [446]. As a result of exposure to antibiotics, the level of resistance against antibiotics among bacteria belonging to the normal intestinal flora of humans and animals increases. These bacteria not only constitute an enormous reservoir of resistance genes for pathogenic bacteria, but also the level of resistance within the endogenous flora is considered to be a good indication of the selection pressure exerted by antibiotic use in that population and for the resistance problem to be expected in pathogens[449].

When animals become colonised with resistant organisms, these organisms can eventually reach humans through the food chain by direct contact or contamination of water or crops from animal excreta[488].

Animal to human transmission of antibiotic resistant pathogens and the role of antibiotic use in food animals in the emergence of these bacteria are controversial subjects[408]. However, there are two potential mechanisms of resistance transfer; (i) resistant strains spread, and (ii) horizontal gene transfer (HGT).

### 1.5.1.1 Resistant strain spread

Most investigations on the transfer of resistant bacteria from animals to humans concern Gram negative food infections caused by bacteria such as *Salmonella* spp and *Campylobacter* spp.

#### 1.5.1.1.1 *Salmonella* spp

Non-typhoid Salmonella infections are the principle cause of food poisoning world wide with an estimated annual incidence of 1 billion cases and 3 million deaths[55]. In the USA alone Salmonella strains cause an estimated 1.4 million infections[291], mostly in children and the elderly, with 16,000 hospitalizations and approximately 600 deaths each year[135,170,324]. The economic costs due to food-borne Salmonella infections in the USA are estimated at $2.4 billion per year[150].

While the majority of infections result in asymptomatic or self-limiting diarrhoeal illness[500], severe, life-threatening septicaemias and other deep-seated infections do occur, particularly among the immunocompromised[484], neonates and the elderly[480] and require effective antibiotic therapy[50]. Despite legislation targeted at controlling the overall use of antimicrobials in food-producing animals, in recent years there have been significant increases, within developed countries, in the occurrence of antimicrobial resistance in non-typhoidal *Salmonella* spp including multidrug-resistant (MDR) *S.*

*enterica* serovars Typhimurium strain DT104[297] and Newport[347,429]. Of particular concern is the development of resistance to key antimicrobials such as extended spectrum cephalosporins (ESC)[114] and the fluoroquinolones because of the importance of these antimicrobials in the treatment of systemic infections in children[50] and adults[324], respectively. Cases of infection in humans caused by both ESC and quinolone-resistant *Salmonella* spp acquired from animals and subsequent treatment failure have been reported[135,297].

The results of a study by Winokur *et al* on animal and human MDR Salmonella concluded that while they could not definitively prove the spread of MDR Salmonella from an animal source to humans the genetic relatedness of the plasmids identified and their prevalence in animal isolates was at least suggestive[485]. Fey *et al* investigating a ceftriaxone-resistant Salmonella infection in a child whose father had treated cattle infected with the same clone concluded that while the infection was transmitted from cattle to child the route of transmission was not through the food chain and remained unresolved[135]. Mølbak *et al* have reported an outbreak of MDR *Salmonella enterica* serovar Typhimurium DT104, in humans, from contaminated pork. Those infected had contracted the organism through occupational contact and consumption of contaminated meat, there was also one case of nosocomial transmission in which the patient died. They identified the likely reservoir of the outbreak clone in two swine herds that had been processed at the incriminated abattoir[297]. In contrast, a review by Piddock concluded that although Mølbak *et al* had shown that the same strain type as in the outbreak could be isolated from two pig farms, there was no evidence of quinolone use on the farms and that the pigs may have acquired the quinolone resistant strain from another, source possibly human[338]. While Mølbak proposed that the strain had been

transferred to those infected via the food chain, Piddock suggests that, although unlikely, those infected had acquired their strains from another source and that it was a coincidence that the strain types were the same. While it is accepted that *Salmonella enterica* serovar Typhimurium DT104 is a zoonotic pathogen and humans usually become infected by eating contaminated food, often poultry, it is not widely accepted that quinolone-resistant strains of this same serovar and type are transmitted through the food chain[338].

#### 1.5.1.1.2 *Campylobacter* spp

Infections caused by *Campylobacter* spp., particularly *Campylobacter jejuni* and *Campylobacter coli*, are among the commonest causes of human gastroenteritis in the world[273]. Campylobacterioses are zoonotic diseases with domestic animals such as poultry[101,337,403], pigs[99,328,364] and cattle[25], and companion animals such as cats and dogs acting as reservoirs. As these organisms may be transferred from animals to humans through the food chain, the emergence of antibiotic resistance, especially fluoroquinolone resistance in *Campylobacter* spp is of particular concern[66]. There are two opposing views on the use of fluoroquinolones in animals and resistance detected in *Campylobacter* spp isolated from human infections. Engberg *et al* have reported that fresh raw meat, especially poultry, is a major source of campylobacter infection and that the emergence of fluoroquinolone resistance in humans has often coincided with or has followed the approval of fluoroquinolones in veterinary medicine[119,120]. Gaudreau *et al* has reported that many European countries and the USA have shown an increase in the levels of fluoroquinolone resistance in *Campylobacter* spp, especially *C. jejuni*, since their introduction, between 1987-1993 in Europe and 1995 in USA, for the treatment and prevention of disease in poultry [149].

Endtz *et al* have proposed that ciprofloxacin resistant campylobacter emerged in poultry after quinolones were used in the treatment of infected flocks and that these bacteria entered the food chain and have caused infections in humans[118]. In contrast, prior to the introduction of the fluoroquinolone, enrofloxacin, into veterinary medicine fluoroquinolones were widely used in human medicine in a number of countries without the emergence of resistance in *Campylobacter* spp in humans[199]. However, Ishihara *et al* reported that a fluoroquinolone resistant campylobacter was isolated, in Japan, from a diarrhoeal patient in 1989 two years before the introduction of enrofloxacin into veterinary medicine[199].

#### 1.5.1.1.3 Enterococci

In addition to being a member of the normal faecal flora of humans and animals enterococci have the ability to cause a wide range of serious nosocomial infections[204]. Of particular concern has been the acquisition of resistance to many antimicrobial agents, especially the glycopeptides. Glycopeptide-resistant enterococci (GRE) were first discovered in Europe in 1986[250] and in the USA in 1987[365]. It has been proposed that the emergence and spread of GRE in Europe has been due to the use of avoparcin, since the mid 1970's, as an AGP in animal husbandry[359]. GRE frequently contaminate meat and meat products. These organisms have been shown to survive gastric passage and colonise the gut of humans[160]. However, the role that non-human sources and reservoirs may play in the spread of *Enterococcus* spp remains controversial[184].

The finding of genetically related GRE isolates in food animals, meat products[233], and hospital in and outpatients suggest that clonal transmission between animals and humans can occur and may contribute to colonisation and subsequent infection in

humans[377]. van den Bogaard *et al* [448] and Stobberingh *et al* [413] have both reported indistinguishable GRE in the faeces of Dutch turkey farmers and their flocks. Lu *et al* reported an outbreak of *E. faecium* infection in pigs and farmers with indistinguishable pulsed field gel electrophoresis (PFGE) patterns[272]. Jensen *et al*, on the basis of analysis of *van*X variants on *Tn1546* in *E. faecium* from humans, pigs and chickens, argue that the spread of entrococci is from animal to man and not vice versa[203]. In contrast, Song *et al*, based on PFGE and polymerase chain reaction (PCR) detection of the glycopeptide resistance genes *vanA* and *vanC*, reported that there was a low incidence of vancomycin resistant enterococcus (VRE) colonisation in humans, despite a high recovery rate from raw chicken meat, and that there was little evidence to suggest that the transmission of VRE from animals to humans occures[406]. Seo *et al* compared VRE from humans, poultry and pigs and reported that there was no difference in the rates of VRE isolation in food animals regardless of whether avoparcin was used[389]. Using repetitive extragenic palindromic PCR (rep-PCR) to compare the genetic relatedness of high level VRE isolated from animals and humans, they reported that isolates from animals were heterogeneous while human isolates showed significant similarity and reported that no genetic relatedness existed between human and animal isolates of VRE[389]

#### 1.5.1.1.4 *E. coli*

*E. coli* are part of the normal micro flora of the gastrointestinal tract of mammals and birds. Certain strains of *E. coli*, O157:H7, O26, O111, O103, O105 and O1 in man and O2 and O78 in poultry, F5 in calves, and O149 from pigs have been associated with gastrointestinal and systemic disease, some being zoonotic[36,68]. Multiply antibiotic resistant non-pathogenic *E. coli* from the gut of animals have been shown to

contaminate meat at slaughter and eggs during laying[446]. However, opinions differ as to whether strains from animals are able to colonise the human intestinal tract. Dupont *et al*[113] concluded that while *E. coli* from animals are able to colonise humans this colonisation is transient and that animals are not an important source of resistant coliforms. Smith[402] also concluded that animal strains of *E. coli* were poorer than human strains at colonising the alimentary tract of humans and that animals are not an important source of antibiotic resistant *E. coli.* Shooter *et al*[395] found significant differences in 'O' serotypes of animal and human *E. coli* and concluded that *E. coli* from animals may differ from those of humans. In contrast, Linton *et al*[266] have shown that antibiotic resistant *E. coli* isolates from chickens were able to colonise the intestinal tracts of human volunteers. Ojeniyi[320] has also reported the direct transmission of antibiotic resistant *E. coli* from poultry to their attendants. van den Bogaard *et al* [446], investigating the antibiotic resistance patterns and clonal relationship of *E. coli* isolates from poultry and humans, concluded that the results of the study strongly indicate that the transmission of resistant clones of *E. coli* from poultry to humans commonly occurs.

### 1.5.1.2 Horizontal resistance gene transfer

Concern over the safety implications of antibiotic-resistant bacteria in foods has also focused on the question of how likely such bacteria are to transfer antibiotic resistance genes to human intestinal bacteria during their passage through the intestinal tract and what might happen to the transferred genes once they enter the colonic flora[315]. Studies have reported evidence of horizontal resistance gene transfer among organisms in soil, in water[92], on the skin and in the intestine of laboratory rodents[27,258,314] and in experimental abcesses[56]. Phillips *et al* proposed that the ultimate defence of those who support the farm-to-clinic hypothesis is that provided animal organisms reach the

human faeces, they need to survive for only brief periods to pass on their antibiotic resistance genes to resident organisms. However, they cast doubt as to whether horizontal gene transfer (HGT) occurs, between organisms of animal origin and those of the human gut flora[336]. It has been well documented that antibiotic resistant bacteria from livestock can and do enter the food supply and have been isolated in meat intended for sale in shops and that these organisms not only pass through but can also colonise the intestinal tract of humans[70,380,480]. A study undertaken by Davison reports that the ability of bacteria to exploit new environments and to respond to new selective pressures can be more easily explained by the acquisition of new genes by HGT rather that the sequential modification of gene function by the accumulation of point mutation [92].

Several studies have shown that the transfer of antibiotic resistance genes, by HGT, can and does take place between a variety of Gram-positive and Gram-negative bacteria. Nikolich *et al* investigated tetracycline-resistant *Bacteroides* spp, a major constituent of the human gut flora, and *Prevotella ruminocola*, the predominant genus of the microflora of ruminants and other farm animals, and concluded that extensive natural gene transmission had occurred and that among the exchange events was the recent transfer of a *tet*Q allele between members of these two genera[315,368]. Oppegaard *et al* has reported the horizontal transfer of a multi-drug resistance plasmid between *E. coli* in cattle and humans[325]. In a retrospective study Salyers *et al* sequenced resistance genes, *tet*Q and *erm*F, found in colonic bacteria. They concluded that since the 1970's there had been a significant increase in the carriage of these genes in *Bacteroides* spp. Sequence analysis of these genes few sequence differences in all the *tet*Q and *erm*F genes examined, indicating horizontal transfer of these genes, in the human colon,

possibly from an animal source over a time period that is short in evolutionary terms[368]. They also found the HGT of macrolide resistance genes *erm*B and *erm*G genes previously exclusively found in Gram positive bacteria, had entered *Bacteroides* spp. between 1970 and 1990[368].

In Germany, Witte found the HGT of plasmid mediated streptothricin resistance, a drug used as an AGP in pigs with no human use, in *E. coli* from urinary tract infections and in *Salmonella* spp and *Shigella* spp from cases of diarrhoea in humans[490]. Hunter *et al*[194] and Chaslus-Dancla *et al*[69] have both reported the horizontal transfer of apramycin resistance, conferring cross resistance to gentamicin, kanamycin and tobramycin, encoded by aminoglycoside 3-N-acetyltransferase IV (*aacC4*) between bacteria, both commensal and zoonotic pathogens, from animals to humans. Salauze *et al* provide evidence of the horizontal transfer of hygromycin B resistance, linked to an *aacC4* as an operon with an association with *IS140* and conferring cross resistance to other aminoglycoside antibiotics, encoded by hygromycin B- phosphotransferase 4 (*hphB*) between animal and human isolates of *E. coli*, *Salmonella* spp, *Klebsiella* spp and *Serratia* spp[367].

In conclusion, both HGT and clonal expansion occur and contribute to the spread of antibiotic resistance; however, the extent to which each mechanism contributes to the observed rise in antibiotic resistance is unclear. HGT may be sufficiently common to drive the dissemination of antibiotic resistance alone, with clonal expansion working only to amplify the genes within individual organisms. HGT may be a rare occurrence with clonal expansion of bacteria with antibiotic resistance genes accounting for the growth in the levels of antibiotic resistance [41].

## 1.6 *Staphylococcus aureus*

### 1.6.1 History and taxonomy

Staphylococci are a ubiquitous and diverse group of organisms that are widely distributed in a variety of natural environments[38]. Natural populations are associated, in high concentration, with the skin, skin glands and mucous membranes, of warm blooded animals. Staphylococci are also found in the genito-urinary and upper-respiratory tracts, in animal products, and other environmental sources such as soil, sea water, fresh water, dust and air and have the ability to persist in a variety of adverse environments[177].

The first reports of staphylococci were published by Recklinghausen, Waldeyer, Birch-Hirschfeldt, Klebs and Hueter in 1871 and 1872. They reported the presence of round elements in pus, kidney, blood and abscesses of infected patients. In 1880 Pasteur published the results of a study into the causes of furuncles, osteomyelitis and puerperal fever. From cases of puerperal fever he isolated "en longs chapelets de grains" now classified as streptococci and in furuncles and osteomyelitis he reported the presence of "le petit vibrion pyogénique" or staphylococci by modern classification [343].

The name staphylococcus was first used by the Scottish surgeon, Alexander Ogston in 1881, from the Greek nouns "staphylé", a bunch of grapes and "coccus", a grain or berry, as a common name for cluster-forming cocci[1]. However, it is Rosenbach who is acknowledged for introducing the common name of staphylococcus in 1884. Prior to this many other bacteriologists had used the terms monad, micrococci or microsporon to describe this group of organisms seen in diseased tissue [234].

Rosenbach first assigned staphylococci into species based on colony colour. The yellow-orange colony type he called *Staphylococcus pyogenes aureus (Staphylococcus aureus)* and the white colony type *Staphylococcus pyogenes albus (Staphylococcus albus)*. This characteristic was also recognised and suggested by Elek, Verneiul and Ogston. A third staphylococcal species, *S. citrus*, a lemon coloured colony type, was added by Passet in 1885. This method of classification proved unacceptable mainly because pigmentation is genetically unstable in many staphylococcal strains[343].

Elek expressed the opinion that the medical bacteriologist is not very concerned as a rule with classification or nomenclature. To him the broad division into pathogens and non-pathogens overrides other considerations, and further subdivision matters only if it aids the recognition of a pathogen or enables him to trace the source of infection. The biologist aims to fit the pathogen into the general order of living creatures, caring little about disease and the microbial features that enter into its causation. The classification of staphylococci is much bedevilled by this fundamental difference in aim [234].

By the early 1900's most bacteriologists were aware that staphylococci were present in a variety of natural environments as well as resident on most animal species including humans. At this time most weight was placed on the identification of *S. aureus* from the commensal staphylococcus, *S. albus.* Methods used to identify *S. aureus* were at this time unreliable even though the coagulase test was available. However, this test remained un-noticed until 1925 when Von Darányi indicated the value of the coagulase test in the identification of *S. aureus*[90]. Although the coagulase test was available and by the mid 1920's was being promoted its use was not routinely accepted until the 1930's when the link between coagulation of plasma and pathogenicity became generally

accepted[343]. Evans in 1948 reported the observation that *S. aureus* were able to ferment mannitol anaerobically. This reaction together with the coagulase test was used in the 7th edition of Bergey's manual to identify *S. aureus*. The genus staphylococcus was recorded as a member of the family *Micrococcaceae* and comprised of only two species *S. aureus* (coagulase positive and fermented mannitol anaerobically) and *S. epidermidis* (coagulase negative and unable to ferment mannitol) [1].

In the late 1800's bacteriologists were separated into two camps; those who wished to classify staphylococci on the basis of pathogenicity, and those who wished to place staphylococci into a universal order of bacteria. At this time all cocci were incorporated into a single family the "coccaceae" with staphylococci, streptococci and gonococci in one sub-division and the non-pathogenic micrococci in another. By the early 20th century the *coccaceae* were split into two sub groups; the *paracoccaceae* which included staphylococci and streptococci and the *metacoccaceae* that was composed of micrococci and sarcinas [486]. The separation of staphylococci from micrococci, although discovered and reported between 1882 and 1900 was not generally accepted until the 1950's when Evans proposed that staphylococci and micrococci could be separated based on there requirement for oxygen. Those species that were facultative in there requirement for oxygen were placed in the genus staphylococcus and those that were obligate aerobes were classified as micrococcus[1]. In 1965 the first meeting of the subcommittee on staphylococci and micrococci, introduced by the International Association of Microbiological Societies, met and set the criteria for the separation of staphylococci from micrococci based on the ability of staphylococci to produce acid anaerobically from glucose in a peptone-yeast extract medium using bromo-cresol purple as a pH indicator[234].

Since 1965 the taxonomic position of staphylococci remained in flux. However the introduction of newly developed genetic investigations allowed taxonomists to clearly demonstrate that although staphylococci and micrococci are both catalase positive, Gram-positive cocci, their guanine and cytosine content as a molar percentage is significantly different with staphylococci having a G+C mol% content of 30-39 while members of the genus micrococcus had a much higher G+C content of 63-73 mol% indicating that these genera are not closely related[234]. Numerous other testing methods, including haemolytic activity[178], digestion of gelatine and milk[234] serological examination[235], agglutination[108], precipitation reactions, cell wall analysis[91], nucleic acid hybridization studies[93], comparative analysis of 16S rRNA sequences[421], rRNA gene restriction site polymorphism analysis[71] and Polymerase Chain Reaction (PCR) based methods[283,495] have also been used in an attempt to clarify staphylococcal taxonomy. Kloos *et al* and Schleifer *et al* have both reported and stressed that the genera staphylococcus and micrococcus have no close relationship to one another, according to phylogenetic concepts, however to date both remain in the one family the *Micrococcaceae* together with stomatococcus and planococcus[234,371].

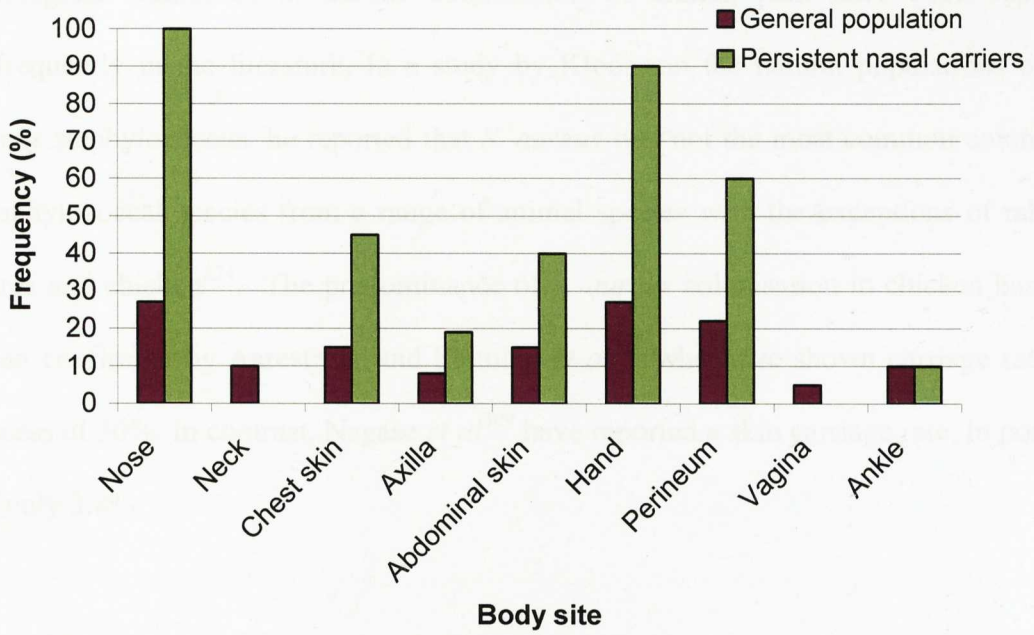
The 7th Edition of Bergey's Manual of Systematic Bacteriology, published in 1957, described only two species of staphylococci. It wasn't until 1974 that the 8th Edition listed a third species *S. saprophyticus* although this species had been described and proposed in 1951[390]. Since 1975 the genus staphylococcus has undergone extensive revision with investigations into new environments and different animal hosts brining about the addition of new species to the genus staphylococcus [177].

### 1.6.2 *S. aureus* colonisation

#### 1.6.2.1 Human colonisation

*S. aureus* colonises the skin and mucosae of humans and several animal species. Although numerous body sites can be colonised a study by Kluytmans *et al*[236] has reported that the desquamated epithelial cells of the anterior nares (vestibulum nasi) represent the dominant ecological niche for *S. aureus* as the elimination of nasal carriage leads to loss of carriage in other areas. The rate of *S. aureus* nasal carriage in the general population ranges from 30% to 70%[264]. Based on longitudinal studies nasal carrier status has been divided into three categories: persistent carriage, intermittent carriage, and non-carriage. These studies have shown that approximately 20% of the general population (range 12-30%) are persistent nasal carriers, 30% (range 16-70%) are intermittent carriers, and 50% (range 16-69%) are non-carriers. Persistent carriers are often colonised by a single *S. aureus* strain over long periods while intermittent carriers may carry different strains over time[477]. The *S. aureus* load is also higher in persistent nasal carriers and increases further if carried both in the nose and perineum. Carriage of *S. aureus* in the nose appears to play a key role in the epidemiology and pathogenesis of infection[236], a view strengthened by showing a correlation between colonisation densities of *S. aureus* at the carriage site and the risk for the development of infection[479]. Host characteristics have been shown to substantially co-determine *S. aureus* carrier state and that an optimal fit between the host and bacteria is essential[236]. This view is further supported by a report by Wertheim *et al* which has shown that *S. aureus* extra-nasal carriage rates are higher in nasal carriers than in non-carriers (Figure 1.6) and that carriage is also higher in those with immunosuppressive illness such as diabetes, HIV, and end stage liver and kidney disease[477].

Figure 1.6 *S. aureus* carriage rates per body site in adults[477]




Studies comparing carriage and infecting isolates of *S. aureus* have demonstrated that individuals are usually infected by their own carriage strain, and temporary eradication of carriage following the use of topical mupirocin has been shown to reduce infections[236]. Also carriage rates vary between ethnic groups, with increased rates in Caucasians especially men and are age dependent[163]. Prevalence rates of *S. aureus* colonisation in neonates and children are consistently higher than those from adults and may reach 50-100%[349]. Peacock *et al*[331] report that infant nasal carriage rates varied significantly with age, falling from a high of 40-50% during the first eight weeks to 21% at six months. Kloos has also shown an age dependent variation in nasal carriage reporting that in pre-adolescent children the nasal carriage rate for *S. aureus* is often higher than in adults and that the populations are often more dispersed over the child's body[234].

### 1.6.2.2 Animal colonisation

Ecological studies of *S. aureus* colonisation of animal skin have been reported infrequently in the literature. In a study by Kloos, on the natural populations of the genus staphylococcus, he reported that *S. aureus* was not the most common colonising Staphylococcal species from a range of animal species with the exceptions of rabbits, hares and chicken[234]. The predominance of *S. aureus* colonisation in chicken has also been confirmed by Aarestrup[5] and Shimizu *et al*[393] who have shown carriage rates in excess of 30%. In contrast, Nagase *et al*[309] have reported a skin carriage rate, in poultry, of only 3.8%.

Kloos has also reported that cattle are able to support populations of *S. aureus* on udders, in teat canals and in milk but was infrequently recovered from the nares. In sheep and pigs *S. aureus* populations have been identified colonising both the nares and udders[234] with one study recording a skin colonisation rate of 41.7% in pigs[309]. In a study by Shimizu *et al*, the *S. aureus* colonisation rate in horses was 30% and Loeffler *et al*, while not giving any specific figure reported that *S. aureus* was the predominant species isolated from horse[393,270]. In contrast, Kloos concluded that horses could carry populations of *S. aureus* but their residency status remained unclear[234]. As early as 1959, Mann reported a *S. aureus* nasal carriage rate of 23% in dogs and proposed that the household pet was an important carrier, and therefore reservoir of *S. aureus* infection for man[281]. Other reports have indicated that dogs are able to support nasal populations of *S. aureus* that have been shown to be closely linked to human dog handlers[280]. A more recent and worrying development has been the isolation of methicillin resistant *S. aureus* (MRSA) in companion animals which have acted as a reservoir of infection to their owners[280,168,431].

### 1.6.3 *S. aureus* strain characterization

#### 1.6.3.1 Characterization of human strains

The ability to discriminate between different strains of a bacterial species has several applications, including investigations of bacterial species evolution[241,321], bacterial virulence[265], and disease transmission[271]. Naturally, an understanding of the dynamics of spread and an identification of transmission or outbreaks are of interest not only to epidemiologists but also to microbiologists and those involved in patient and animal management. Staphylococcal strains may vary considerably in virulence and epidemiological potential[265]. To control the spread of staphylococcal infection the sources of contamination and mechanisms of transmission need to be identified. Studies of the pathogenicity and epidemiology of staphylococci depend on the availability of typing schemes that can differentiate between strains. Numerous phenotypic and genotypic methods have been used for typing *S. aureus* isolates (Table 1.4)[197,381,175,,286,104,362,88,237,444,47,450,300,245,122].

Table1.4 Phenotypic and genotypic methods of *S. aureus* characterization

| Phenotypic methods | Genotypic methods |
|---|---|
| Antibiograms | Multilocus enzyme electrophoresis (MLEE) |
| Biotyping | PFGE |
| Bacteriophage typing | Multilocus restriction fragment typing (MLRFT) |
| Plasmid profiling with restriction endonucleases | Multilocus variable number tandem repeat analysis of polymorphisms (VNTR) |
| | Amplified fragment length polymorphism (AFLP) |
| | Random amplification of polymorphic DNA (RAPD) |
| | Arbitrary primed PCR |
| | Sequence of the 16S rRNA |
| | Endonuclease digestion of the variable region of the *coa* gene |
| | Protein A gene polymorphism |
| | Coagulase gene polymorphism |
| | Sequence of the protein A (*spa*) gene |
| | ITS-PCR |
| | Multilocus sequence typing |

The discriminatory powers of most of the phenotypic methods of characterization have been shown to be restricted and ambiguous. This was also reported by Tenover *et al* who concluded that several studies suggest that phenotypic markers, such as biotyping, antibiotic susceptibility patterns, and bacteriophage typing, are more likely to change over time than those detected by molecular typing schemes, such as PFGE, MLST, and *spa* typing [423].

Molecular typing of *S. aureus* has been used to examine both the long-term or global epidemiology and short-term or local epidemiology. Understanding the genetic structure of a global population over time gives insights into the evolution of bacterial lineages and transmission dynamics and also provides a framework with which to study bacterial pathogenesis[329]. Bacterial population analysis of *S. aureus* indicates that phylogenetic lineages are not always randomly distributed within clinical isolate populations. Within the *S. aureus* species discrete lineages or subtypes exist due to the selective pressures imposed by antibiotic use and other ill defined environmental factors[307]. In recent years, the use of nucleotide sequence variation at multiple housekeeping loci have become increasingly popular for strain characterization, as it has the advantage of inferring levels of relatedness between strains and the reconstruction of evolutionary events[133]. For example, the majority of MRSA have expanded clonally and globally upon the acquisition of the *mec* determinant and have been shown to have methicillin-susceptible *S. aureus* (MSSA) counterparts with identical multilocus sequence typing (MLST) types[49]. This indicates that methicillin resistance probably arose in genetic backgrounds that were inherently fit with a decreased drug susceptibility allowing an extension of the niche occupied by the bacterium to include the antibiotic rich hospital environment[122]. MLST has identified five major pandemic clonal lineages of *S. aureus* that are widely

distributed and account for a large proportion (70%) of isolates, indicating that they represent successful lineages in terms of ability to cause infection, to persist, and to spread from one geographic site to another, including across continents[355]. These five major pandemic clonal complexes (CCs) or lineages are CC5 (New York/Japanese, South German and Paediatric clones), CC8 (Archaic, Brazilian and Hungarian clones), CC22 (EMRSA15 clone), CC30 (EMRSA16 clone), and CC45 (Berlin clone) (Figures 1.7, 1.8, 1.9, 1.10, 1.11)[123]. Additionally, CC1 represents a lineage strongly associated with emerging community acquired MRSA infections. A number of other lineages can be found among the *S. aureus* species that are never or occasionally MRSA or infrequently isolated in general[217].

Figure 1.7 Evolutionary origins of CC5 genotypes. Arrows indicate either the acquisition of SCC*mec*, or a change of Multilocus Sequence Type[123].

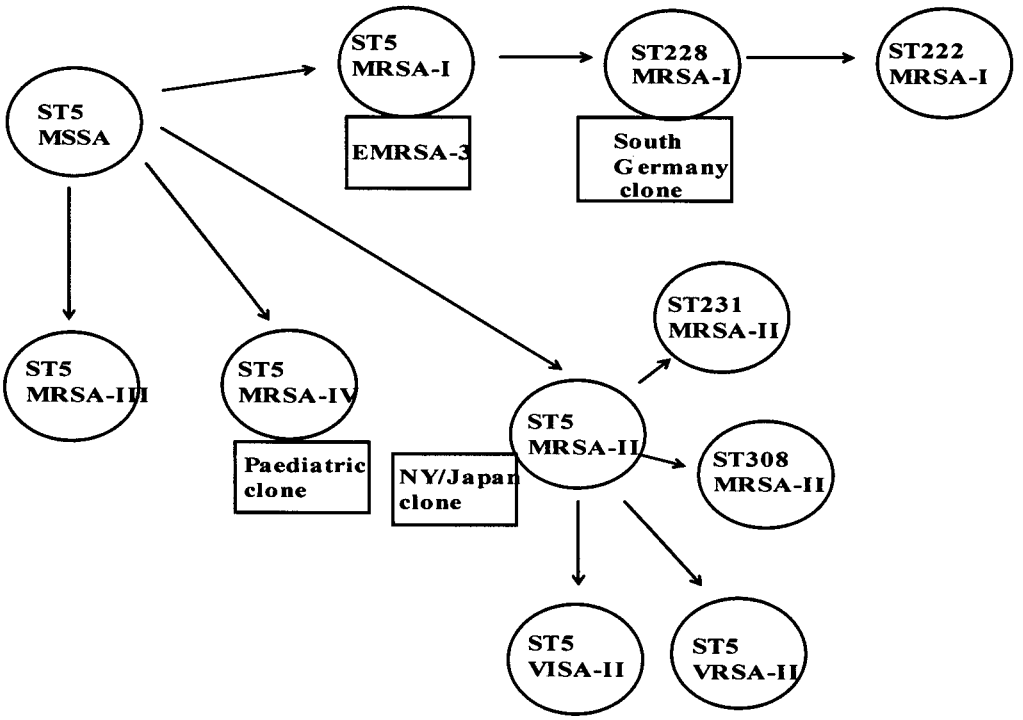


Figure 1.8 Evolutionary origins of CC8 genotypes. Arrows indicate either the acquisition of SCC*mec*, or a change of ST[125,157,356]

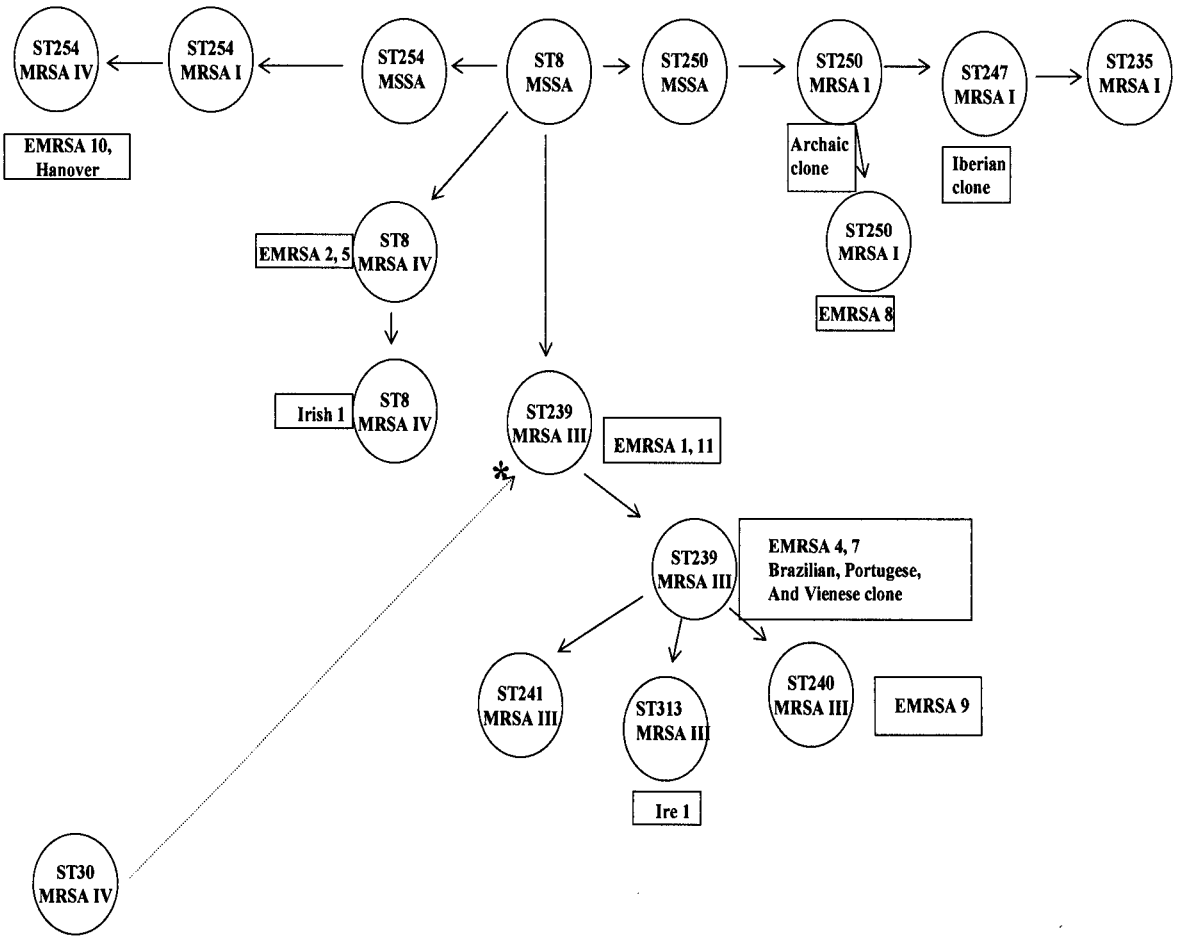


Figure 1.9 Evolutionary origins of CC22 genotypes. Arrows indicate either the acquisition of SCC*mec*, or a change of ST[356]

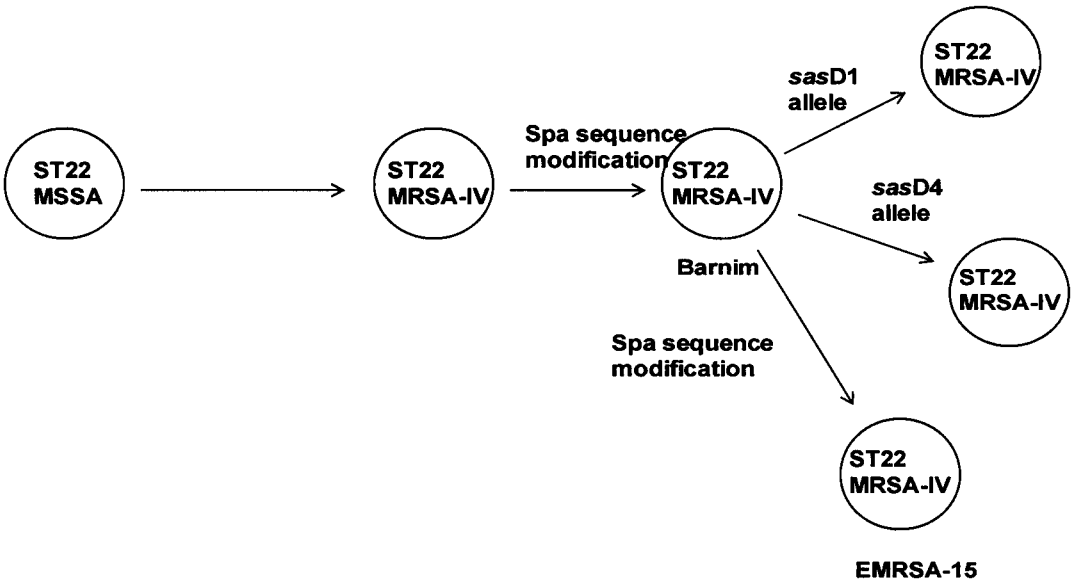



Figure 1.10 Evolutionary origins of CC30 genotypes. Arrows indicate either the acquisition of SCC*mec*, or a change of ST[124,125,132]

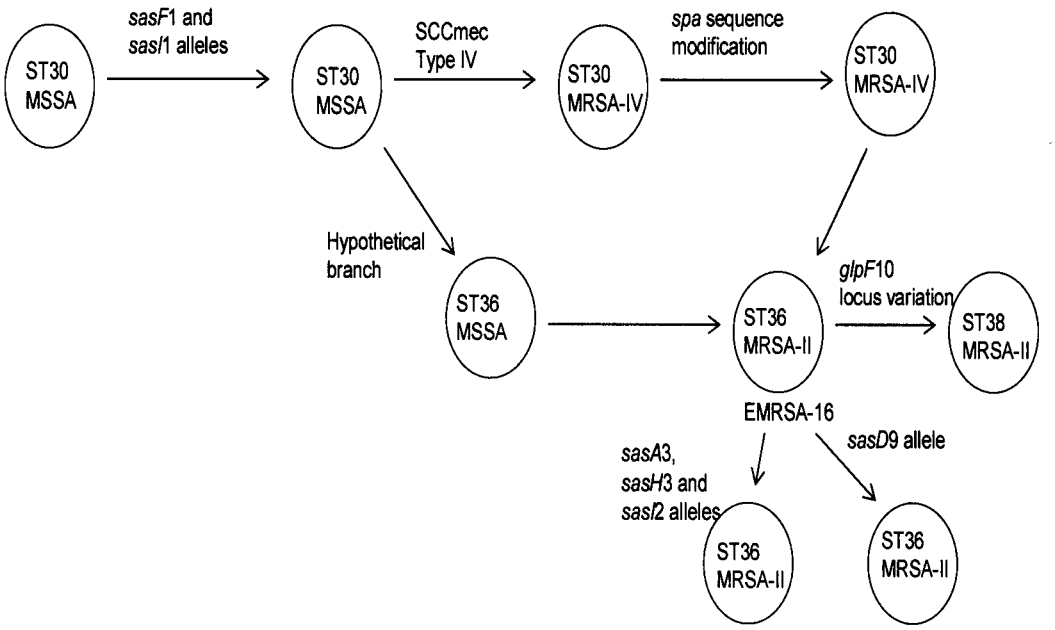
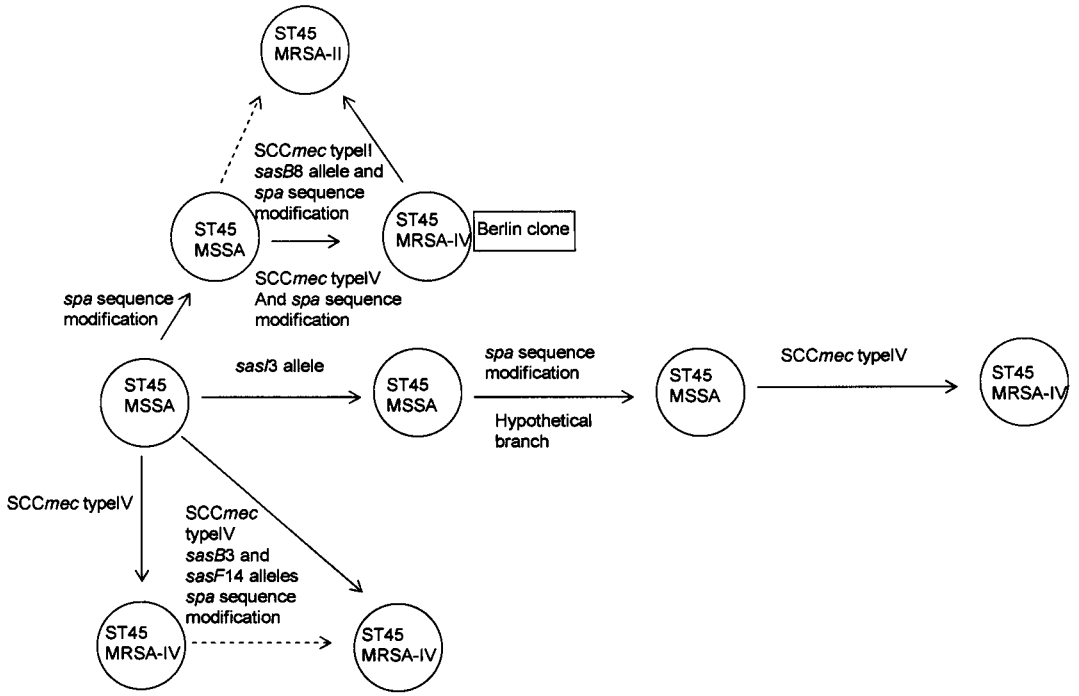


Figure 1.11 Evolutionary origins of CC45 genotypes. Arrows indicate either the acquisition of SCC*mec*, or a change of ST[356].



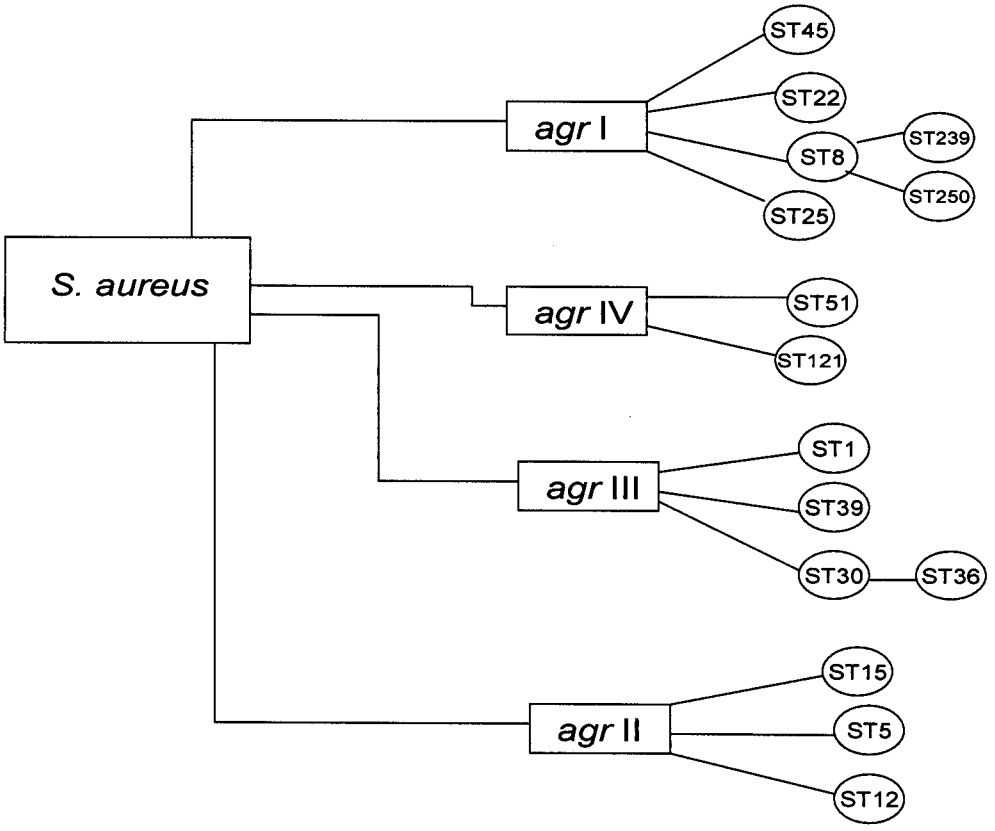

Although genes encoding essential housekeeping functions are commonly viewed as the most reliable markers, the precise importance of gene function in predicting the utility of intra-species markers has not been systematically investigated[82].

Clones of *S. aureus* have been shown to arise 15 times more frequently by point mutation of the housekeeping genes than by homologous recombination[132]. However, Robinson *et al*, using MLST and sequence data from an additional eight surface protein encoding genes (seven *sas* genes and the gene that encodes immunoglobulin G binding protein A (*spa*)) has shown that three significant recombination events have occurred within the five major MRSA CCs. These recombination events were between ST8 and ST30 to form ST239* (Figure 1.8), ST30 and ST145/ST10 to form ST34 and ST39 and

ST145/ST10 to form ST42[357]. Khun *et al* have also shown homlogous exchange events in core and acessory gene alleles between isolates of distant phylogenetic lineage[241].

In a study by Wright *et al* they report that genotyping of *S. aureus* strains by five different methods (PFGE, MLEE, MLST, *spa* and *coa* typing) genotypes that were so strongly correlated with *agr* types that the former could be used to predict the latter unequivocally (Figure 1.12). They hypothesise that there is a direct relationship between *agr* groups and clone phylogeny based on the finding that with exceedingly rare exceptions, no MLST pattern occurred in more than one *agr* group[491].

Figure 1.12 Evolutionary divergence of the *agr* locus[491]



The hypothesis that *agr* groups delineate fundamental subdivisions within *S. aureus* was rejected by Robinson *et al* who mapped *agr* groups on to a phylogenetic tree based on the sequence analysis of the seven MLST housekeeping genes and seven additional genes that encode putative surface protein genes (SAS genes) by Shimodaira-Hasegawa and parametric bootstrap tests. They were unable to reject the hypothesis that *S. aureus* was subdivided into two groups consisting of multiple CCs and multiple *agr* groups[358]. Kuhn *et al* compared the partial sequence data from the seven MLST housekeeping genes, six core adhesins (*clfA*, *clfB*, *fnbA*, *map*, *sdrC*, and *spa*) and four accessory adhesion genes (*ebpS*, *fnbB*, *sdrD*, and *sdrE*). Gene trees constructed from concatenated sequences of these core and highly variable accessory genes have shown that the variable genes were at least as informative for phylogenetic reconstruction as the slowly evolving housekeeping genes[241].

Cooper *et al* investigated a diverse range of *S. aureus* by MLST and supplemented this data with a further 33 gene loci. These supplementary genes were grouped into three functional classes: information pathway genes, housekeeping genes and cell envelope/cellular process genes. They also included characterization data of conserved genes of unknown function and orphan genes. These data were concatenated and used to produce an unrooted Bayesian tree[82]. This tree was broadly consistent with the phylogenetic tree produced by Robinson *et al* and confirmed the division of *S. aureus* into two main groups with group 1 being subdivided into groups 1a and 1b[82,358]. In contrast to the MLST data on the evolution of CC30 and CC45 by Enright *et al*[125], data produced by Cooper *et al* suggest that ST30 and ST45 share a common ancestor and conclude that there is little evidence to justify the current emphasis on the use of housekeeping genes for phylogenetic, systematics, or epidemiological studies as there

data suggests that the MLST genes for *S. aureus* rate amongst the poorest phylogenetic markers. They do not advocate changes to the established MLST scheme as it has proved to be successful in understanding the population structure of *S. aureus* and for assigning isolates into particular lineages[82].

A study by Lindsay *et al*, on a collection of *S. aureus* associated with community-acquired invasive disease and carriage in healthy individuals using microarray has shown that genes termed core-variable genes, that encoded known or putative virulence and resistance genes, were able to clearly discriminate distinct lineages that closely matched the MLST clonal clusters CC1, CC5, CC8, CC9, CC12, CC15, CC22, CC25, CC30, CC45, and CC51. They note that MLST is able to detect further differences (STs) within each CC that were not corroborated by microarray and suggest that this may be due to the enormous amount of variation seen in the *S. aureus* genome, so that relying on only a few markers placed increased weight on minor differences[265].

### 1.6.3.2 Characterization of animal strains

There are limited data available on the systematic characterization of the natural population structure of *S. aureus* strains isolated from animal species with the exception of those isolates causing mastitis in cattle. Over the past 40 years bacteriophage and biotyping have been used to discriminate *S. aureus* from cattle[103]. Due to poor reproducibility and an inability to type all isolates, however, these techniques are no longer used and have been superseded by molecular typing methods, including PFGE, MLST, *spa*, binary, and *agr* typing, that are commonly used in the characterization of *S. aureus* from humans[344,401,498].

Cross-infection between animals and humans in the household, veterinary hospital and farm setting has been described. Weese *et al* have shown the cross-infection of horses and humans with strains of MRSA, with indistinguishable PFGE patterns, and concluded that this data provides evidence that horse to horse and horse to human transmission of *S. aureus* is possible[467]. This conclusion is consistent with data published by Malik *et al* where, based on MLST, MRSA isolated from canine sources were shown to be ST239 SCC*mec* type III belonging to CC8, a well recognised human MRSA lineage mainly confined to health care settings, indicating the dog became colonised and infected through a household or other close contact with a human MRSA carrier[279]. These studies contrast with the conclusion of Carter *et al* who indicate that genetic studies have shown that strains of *S. aureus* remain closely associated with their animal host with little transfer between species[63]. This conclusion is consistent with characterization studies of *S. aureus* strains isolated from animals, predominantly cattle, that have shown that relatively few clones of *S. aureus* are responsible for the majority of cases of bovine mastitis and that these clones and their derivatives are adapted not only to the bovine mammary gland but also to the bovine environment and have achieved worldwide distribution[401]. Van Leeuwen *et al* have demonstrated that *S. aureus* strains from humans can cross-infect domestic animals, poultry, pet animals and vice versa, however, it has been determined that separate host specific lineages of *S. aureus* exist and the potential of cross-species pathogenicity of these lineages remains unanswered[452].

Kapur *et al*, have also concluded that while cattle herds could be colonised by several distinct *S. aureus* clones and that some of these clones are closely related to isolates recovered from humans many were not suggesting host specialisation. They concluded

that the successful transfer of *S. aureus* between humans and animals under natural conditions was limited[216].

Studies by Smith *et al*, using MLST, have independently reported that the majority of cases of bovine mastitis caused by *S. aureus* clustered within a single clonal complex (CC97) concluding that this constitutes a host adapted *S. aureus* strain[400,401]. This hypothesis of host adaptation was further developed by Smith *et al* where analysis of CC97 diversification suggested that there was a roughly equal chance of housekeeping gene alleles changing by point mutation and recombination indicating an altered population structure of bovine *S. aureus* compared with that of human isolates where point mutation is more frequently involved in clonal diversification[401]. Host specific, host adapted or tissue adapted *S. aurueus* strains have also been reported by Larsen *et al*, using phage and ribotyping[246], Gilot *et al* using *agr* restriction fragment length polymorphism (RFLP)[156], Riamundo *et al* using RFLP of the 3' end of the *coa* gene in bovine strains[344], Rodgers *et al* using biotyping and PFGE in poultry strains[360], and by Katsuda *et al*, Schlegelová *et al*, and da Silva *et al* using RFLP of the 3' end of the *coa* gene in caprine, and bovine strains[218,370,398]. Also, in a study by Strommenger *et al* they report the isolation of an MRSA ST22 (*spa* type 032) from an animal with a similar PFGE restriction pattern to those of human strains and suggest that these animal strains represent a sub population of ST22 that may have adapted to pet animals[415].

### 1.6.4 Host specificity

The concept of host restriction/adaptation/specificity is not new and has previously been reported in staphylococci with *S. hyicus* causing disease only in pigs[471], *S. intermedius* causing disease in dogs[168], and *S. lentus* preferentially colonizing sheep, and goats[234]. Host specificity has also been reported among serotypes of *Salmonella* spp, producing disease in their natural hosts, including *S. typhi* (human/chimpanzee), *S. gallinarum* (avian), *S. abortusovis* (sheep), *S. typhisuis* (pig), *S. abortusequi* (horse), serovars of *S. choleraesuis* (pig), and *S. dublin* (cow)[442]. The host specific adhesins (K88, 987P, and K99) of *E. coli* are associated with a relatively small number of O serogroups (O6, O8, O9, O15, O20, O25, O45, O63, O78, O101, O138, O141, O147, O149, and O157) that cause disease specifically in piglets, calf, and lamb[225]. A study by Willems *et al* has shown the existence of host specific vancomycin resistant *Enterococcus faecium* genogroups that are associated with particular hosts and environments[483]. However, Heuer *et al* doubt Willems conclusion as it implies that hospitalized patients and community based humans be regarded as different hosts[185]. Although the precise mechanisms of host adaptation have not been elucidated it has been suggested that host specific serotypes have adapted to the physiology of their natural hosts and developed a means to capitalize on it[442].

The evolutionary processes in *S. aureus* restricting or expanding invasiveness in different hosts are ill defined[137]. However, Peacock *et al* suggest that the numbers and combinations of certain virulence genes may be significant contributors to pathogenic potential[332]. Molecular studies of *S. aureus* strains isolated from human patients and bovine mastatic milk have indicated that such strains differ and that bovine and human reservoirs represent two separate sub-populations of this species that rarely cross

infect[216]. Classification of *S. aureus* isolates by AFLP of the coagulase gene allowed Schlegelova *et al* to describe host specific dominances of genotypes between bovine mastatic strains and human strains[370]. Data from a study by Carter *et al* investigating the relationship between *S. aureus*, based on coagulase gene polymorphisms, from humans and cows was consistant in general with the theory of host specialisation[63]. Host restriction of *S. aureus* has also been shown between poultry and poultry farm personnel by Rodgers *et al* where poultry and humans carried their own specific *S. aureus* biotype and PFGE type with no evidence of cross colonisation[360]. Many *S. aureus* strains have disseminated widely among humans. These same strains have the potential to colonise and infect many different host species. Conversely, host specific and even tissue specific *S. aureus* strains have been identified causing disease in varied host species[497]. It has been suggested that the presence of combinations of virulence factors plays an important role in host and tissue specificity in *S. aureus* colonisation and infection[452]. The effect of these genes has been shown to be cumulative, each independently multiplying the odds of disease. Host factors such as genetic predisposition via one or more susceptibility genes or acquired factors including the presence of intravenous devices or surgical wounds may also play an important role for *S. aureus* colonisation and infection[452].

### 1.6.5 Pathogenicity

While *S. aureus* forms part of the normal bacterial flora of humans and animals it is also responsible for a wide range of conditions. These range from minor infections of the skin to wound infections, bacteraemia, infections of the central nervous system, the respiratory and genito-urinary tracts and infections associated with intravascular devices and foreign bodies in humans and is also one of the most frequent cause of contagious

mastitis in cattle, sheep and goats, leg and joint infections in poultry[57] skin infections in rabbits[181] and a range of infections in wild, pet[422] and sporting animals[387].

### 1.6.5.1 Disease in humans

*S. aureus* is the most common cause of hospital acquired infection, causing clinical disease in 2% of all patient admissions[264]. It is also a major cause of wound infections and has the invasive potential to induce osteomyelitis, endocarditis and bacteraemia which is increasing and has more that doubled across Europe in the last 25 years[319]. The increase in nosocomial infection has coincided with a growing rate of community acquired disease as a proportion to hospital acquired infection. It has been suggested that part of this effect may however be better explained by host susceptibility, due to immunosuppression, and an aging population[292].

Within the community setting, minor infections of the skin caused by *S. aureus* are common while severe invasive disease is relatively rare. Deresinski has reported an increase in the isolation of *S. aureus* causing community acquired cutaneous infection in children[100]. Several outbreaks of community-acquired skin infections have been reported as a result of infection with MRSA that have acquired the Panton-Valentine leukocidin gene (PVL)[303,419]. These new strains are associated with a particularly lethal form of haemolytic pneumonia in children with a mortality rate of 40%[415]. The acquisition of both multiple antibiotic resistance and PVL in *S. aureus* strains that spread rapidly in the community suggests that *S. aureus* is becoming more virulent and of greater concern to medical professionals[231].

Lindsay *et al*[264] have reported that the *S. aureus* genome is comprised of core, accessory and foreign or lost genes. The core genome makes up ~75% of the *S. aureus* genome and is highly conserved with the majority of genes associated with central metabolism and house keeping functions. However, supplementing these genes are genes of the accessory genome, that makes up the remaining ~25%, and while they are non-essential for growth or survival they consist of a plethora of virulence genes implicated in *S. aureus* pathogenicity. The relative importance of host factors versus bacterial virulence determinants in disease pathogenesis is unknown, but it is widely held that bacterial factors including binding proteins, toxins, exoenzymes, enterotoxins and capsule biosynthesis are involved in the process[332]. Many of the virulence factor encoding genes are located in discrete genetic elements that are mobile or were once mobile, are strain specific, and can transfer horizontally between strains[264]. These genetic elements include plasmids, transposons, converting bacteriophages, and, most recently described, pathogenicity islands and resistance islands[275]. A number of regulators are involved in the production of these virulence determinants in *S. aureus*. These include the accessory gene regulator (*agr*) and staphylococcal accessory regulator (*sar*). The *agr* locus has been shown to be required for virulence by several infection models and regulates virulence in concurrence with the transcriptional regulator SarA encoded by the *sar* locus[148,200,392,451]. Evidence has shown that some elements are able to move frequently while others move infrequently if at all[265]. Many of the genetic elements that carry the genes responsible for virulence and resistance functions have been shown to be associated with a limited number of *S. aureus* lineages or clonal types[264]. Therefore the distribution and horizontal gene transfer of these lineages and elements can have important clinical implications.

### 1.6.5.2 Disease in animals

#### 1.6.5.2.1. Disease in poultry

*S. aureus* is the only staphylococcal species considered pathogenic in poultry and is one of the most frequent causes of infection in poultry worldwide[5,57,35,5,220,340].The most common infections caused by *S. aureus* in intensively reared poultry are those affecting bones, tendon sheathes, leg joints, skin, sternal bursa, yolk sac, heart, vertebrae and eyelid. Granulomas of the liver and lungs and septicaemia especially in laying birds, during periods of hot weather, are also common and may resemble fowl cholera[5,220]. These conditions have been shown to be closely associated with poor management conditions resulting in moist or wet litter that facilitates skin entry of *S. aureus*.

#### 1.6.5.2.2 Mastitis in cattle

*S. aureus* is one of the major intramammary pathogens isolated from cattle with subclinical and clinical mastitis, leading to significant economic loss to the dairy industry due to rejected milk, reduced milk quality, early culling, drug costs, increased veterinary expenses, and labour costs to the farmer[405]. Infected cows' udders are the main reservoir from which *S. aureus* is transmitted to other cows in the herd, and prevention of transmission from cow to cow reduces mastitis incidence[497]. Many sources of *S. aureus* have been reported, including housing materials and fodder, equipment and air, bovine skin, non-bovine animals, and humans[350]. Teat skin has been suggested as an important reservoir for intramammary infection, while human to bovine transmission has also been proposed[497].

#### 1.6.5.2.3 Disease of other animal species

*S. aureus* has been shown to cause a wide variety of infection in a range of animal species. Mastitis and cutaneous abscess in sheep, goats, and broiler rabbit is often associated with *S. aureus*[39,456,457]. In a study by Hermans *et al, S. aureus* infection in rabbits could be divided into two groups; those caused by low virulence strains resulting in chronic infection, and those caused by high virulence strains resulting in an epidemic spread of disease[181]. An investigation of ovine mastitis caused by *S. aureus* has shown that most cases are caused by a few closely related genotypes, which are widely distributed[456]. These infections, caused by *S. aureus*, have a significant economic impact for those farming these animal species. *S. aureus* has also been reported as the cause of postoperative wound infections in horses[174,387,466], wound infections in dogs and cats[256], and also an infection of the spleen and lymph nodes in a seal[158,318]

### 1.6.6 Antimicrobial resistance

#### 1.6.6.1 Antimicrobial resistance in *S. aureus* from humans

*S. aureus* has always been a stumbling block for antimicrobial chemotherapy, since it has overcome all the therapeutic agents that have been developed in the past 60 years[188]. Penicillin was introduced in 1944 and at that time 94% of all *S. aureus* were susceptible; by 1950 the rate of resistance was over 50%, and by the 1980's the rate of penicillin resistance exceded 90%[268]. Data supplied by the Scottish MRSA reference Laboratory over the period 1997-2003 shows that the most frequently detected resistances among human *S. aureus* are to penicillin, erythromycin, gentamicin, fusidic acid, ciprofloxacin and trimethoprim (Table 1.5). Of all the resistance traits *S. aureus* has acquired methicillin resistance is clinically the most important, as it has provided *S.*

*aureus* with a mechanism that has made all members of the largest and most useful family of antibiotics, the β-lactams, obsolete as therapeutic agents[166]. The emergence and global spread of MRSA has been viewed as a process of accelerated evolution driven by the selective pressure of immense quantities of antibiotics introduced into the hospital environment[86].

While MRSA have been acquired almost exclusively in hospitals, long term care facilities, and other institutional settings, the recent reports of community acquired MRSA has led to the speculation that the epidemiology of *S. aureus* is changing[193]. Unlike hospital strains, which are typically multiresistant, these community-acquired strains are more susceptible to antibiotic groups and often only resistant to the β-lactams. This lack of resistance suggests a community origin, as the antibiotic selective pressure is much lower in this environment[65].

The growing prevalence of MRSA as a cause of serious nosocomial infection has increased the use of the glycopeptide antibiotic vancomycin over the past three decades[143]. As a result, vancomycin-intermediate *S. aureus* (VISA) strains that exhibit two different resistance mechanisms have emerged. The first, reported in Japan in 1996[186] and subsequently in the United States in 1997[425], is believed to be due to a thickening of the cell wall that produces intermediate vancomycin resistance (VISA). There are two types of VISA, firstly VISA with an MIC ≥4μg/ml and secondly hetero-VISA (h-VISA) with an MIC ≤2 μg/ml with a resistant sub-population able to grow at higher vancomycin concentrations. h-VISA develop during continued glycopeptide exposure and are thought to be precursors of VISA[192]. Intermediate vancomycin resistance in *S. aureus* has also now been reported from France, Korea[230], South Africa,

and Scotland, while isolates with h-VISA resistance appear to be a global problem[425]. The second, first noted in 2002, is identical to the mechanism seen in vancomycin-resistant enterococcus, involving the *van* genes[143]. The emergence of MRSA with reduced susceptibility or high level glycopeptide resistance has increased the awareness of the now limited number of therapeutic options for serious *S. aureus* infections.

Table 1.5 Frequency of antibiotic resistance (%) in human *S. aureus* in Scotland

| Antibiotic | Time period | | |
|---|---|---|---|
| | 1997-1999 | 2000-2001 | 2002-2003 |
| Penicillin | 11.7 | 21 | 38.6 |
| Erythromycin | 13.5 | 18.7 | 14 |
| Clindamycin | 4.2 | 0.8 | 4.5 |
| Gentamicin | 1.3 | 14.9 | 1.8 |
| Fusidic acid | 2.3 | 7.6 | 12.3 |
| Chloramphenicol | 0.5 | 0.2 | 0 |
| Ciprofloxacin | 10.4 | 4.7 | 10.5 |
| Trimethoprim | 3.6 | 11.5 | 5 |
| Sulphamethoxazole | 4.4 | 2.5 | 2 |
| Tetracycline | 9.4 | 9 | 14.3 |
| Rifampicin | 0.3 | 0.4 | 1 |

### 1.6.6.2 Antimicrobial resistance in *S. aureus* from animals

Antimicrobial agents are widely used in the treatment of *S. aureus* infection in domestic and pet animals. These agents are similar and in many cases identical to those used in humans, including aminoglycosides, β-lactams, glycopeptides, macrolides, quinolones, streptogramins and tetracyclines. With the exception of *S. aureus* mastitis in cattle, there are only a few studies that have determined the frequency of resistance in *S. aureus* from other animal species[5,158,190].

Penicillins and aminoglycosides have been the most frequently used antibiotics in the treatment of bovine mastitis worldwide and are consequently the most frequently

reported resistance, in *S. aureus*, from bovine intramammary infections[85,306]. However, the frequency of resistance to penicillins and aminoglycosides varies considerably between countries with penicillin resistance rates of between 44% and 71% reported from Brazil, Germany, Ireland, the UK and the USA, while isolates from Germany have been shown to have one of the highest rates of resistance to gentamicin (51%)[476]. In contrast, rates of resistance in bovine isolates from Scandinavia and Japan are low and have been attributed to a more restrictive antibiotic policy in these countries[459].

Mastitis is also a significant problem in domestic sheep and rabbits. A study by Gŏni *et al* of antibiotic resistance in *S. aureus* isolated from sheep and rabbits noted that isolates from sheep were mostly susceptible to the antibiotics tested, including aminoglycosides, chloramphenicol, tetracycline, trimethoprim, and erythromycin, but the majority of the rabbit isolates were resistant to various combinations of tetracycline, streptomycin and erythromycin[158].

A large number of antibiotics have been used for therapy in the production of poultry. Aarestrup *et al* have investigated the frequency of resistance in *S. aureus* from poultry to a range of antibiotics and found no resistance to the aminoglycosides or glycopeptides but a surprisingly high rate of resistance to ciprofloxacin (30%), erythromycin (24%), and tetracycline (47%) [5].

In domestic pet dogs *S. aureus* is a common cause of wound infection with antibiotics used in their treatment. Few studies have investigated the frequency of resistance in canine isolates of *S. aureus*. Hoekstra and Paulton examined 867 isolates and found that 93.4% were resistant to at least one of the antibiotics tested and 67.3% were resistant to

≥2 antibiotics. The most frequently seen resistances observed were to cloxacillin (68.2%), lincomycin (78.4%), penicillin (74.2%), and trimethoprim-sulphamethoxazole (74.4%), while agents such as tetracycline, and the newer quinolones were still effective in the treatment of deep seated wound infections[190].

Methicillin-resistant *S. aureus* is a well known human pathogen and a new emerging problem in veterinary medicine. Cases of animal infection and carriage are increasingly reported worldwide and recent studies have emphasized occupational health implications to both farmers and veterinary staff[168]. E-MRSA 15, the most prevalent human MRSA strain in the UK, has been reported from 9% of dogs in a small animal veterinary practice in the UK[270]. The origin of this strain remains unknown in companion animals although it has been proposed that MRSA isolated from campanion animals originate from humans. Recent reports of the isolation of MRSA (ST398) from pigs, horses, dogs and humans in the Netherlands, Denmark, Germany and Austria are raising concern[95]. The origin of ST398, whether human or animal, is presently unknown although it is generally believed that this *S. aureus* lineage has originated from pigs[193,461]. It is not known whether this strain represents a risk to animal health, but several cases of severe infection in humans have been documented and it may therefore represent a significant threat to human health[168].

### 1.7 Aims and objectives of this study

The primary objective of this study is to examine the influence antimicrobial use in animals may have had on human clinical *S. aureus*.

To test this hypothesis firstly the genetic population structure of isolates of *S. aureus* recovered from animals will be assessed, using phenotypic and molecular techniques, and compared with data from human clinical isolates of *S. aureus* (Chapter 2). Secondly, the genetic relationships and potential host adaptation of selected *S. aureus* strains from animals and humans will be examined using a variety of recognized virulence determinants (Chapter 3). Thirdly, the frequency of antimicrobial resistance in *S. aureus* isolated from animals will be determined (Chapter 4) and finally, the presence and relative frequency of genes encoding tetracycline, and macrolide resistance in animal and human *S. aureus*, the presence of structural variation in the *tet*(K) genes and the transferability of *tet*(K) resistance plasmids from animal strains to *S. aureus* 8325-4 will be investigated (Chapter 5).

## Chapter 2 Strain Characterization

### 2.1 Introduction

Outbreaks of infection caused by *S. aureus* continue to be a problem in human and veterinary medicine[28,494,495]. Staphylococcal strains vary considerably in virulence and epidemiological potential. To control the spread of staphylococcal infection, sources of contamination and mechanisms of transmission have to be identified[498]. Identifying strains among outbreak isolates is a major step in determining the source of the outbreak and in determining subsequent control measures[28,424].

Characterization may be done for taxonomic purposes, phylogenetic analysis directed at determining relatedness of target organisms, delineating steps in the evolution of a target organism, or for the molecular epidemiology of pathogenic strains[82,125].

Pathogenic and epidemiological studies depend on the availability of typing schemes that differentiate between strains belonging to the same bacterial species[498]. Typing methods are a major tool for the epidemiological characterization of bacterial pathogens, allowing the determination of the clonal relationships between isolates based on their genotypic or phenotypic characteristics. Recent technological advances have resulted in a shift from less discriminatory phenotypic typing methods, such as serotyping, biotyping, phage typing, and antibiotic resistance typing, to molecular methods such as restriction fragment length polymorphism (RFLP), pulsed field gel electrophoresis (PFGE), PCR amplification of the 16S-23S intergenic spacer region (RS-PCR), and with the availability of affordable gene sequencing methods multilocus sequence typing (MLST) and *spa* typing[62]. Although these are technically demanding

and more expensive than phenotypic methodologies they are able to generate highly reproducible results and are extremely discriminatory for *S. aureus*[133,301,424]. In considering the subtype of an organism, an evaluation of all available data such as biochemical reaction profiles, serotype, bacteriophage type, antibiotic susceptibility, presence or absence of surface appendages, and virulence factors should be considered with reliance on a single parameter for characterization avoided.

The objective of this part of the study is to assess the population genetic structure of isolates of *S. aureus* recovered from animals using a variety of molecular techniques used to characterize human *S. aureus*, and to determine the genetic relationships among these *S. aureus* isolates and compare these with data from human clinical isolates of *S. aureus*.

## 2.2 Materials and methods

### 2.2.1 Bacterial strains

A total of 261 *Staphylococcus aureus* (233 domestic, wild and companion animal and 28 human clinical) isolates were characterized in this study. Two hundred and thirty three of these isolates, collected between 1996 and 2001, were from three Scottish regions; Grampian, Highland, Southwest Scotland, and from Northern Ireland (Table 2.1), and 28 human MR and MSSA, collected between 2000 and 2005, from across Scotland (supplied by the Scottish MRSA reference laboratory) (Table 2.2).

Table 2.1 Distribution of *S. aureus* isolated from animal from Scotland and Northern Ireland

| Geographic location | Animal source | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Livestock | | | | | | | Companion | | | Wild | | | |
| | Chicken | Cow | Lamb | Milk | Pig | Turkey | Unknown | Dog | Horse | Parrot | Partridge | Pheasant | Phillips goose hawk | Saker hawk |
| Grampian | | 45 | | 25 | | | | | | | | | | |
| Southwest Scotland | | 46 | 1 | 38 | | 2 | | | | | | | | |
| Highland | | | | | 7 | | | | | 2 | 3 | 6 | | |
| Northern Ireland | 48 | 1 | | 3 | | | | | 1 | | | | 1 | 1 |
| Total | 48 | 92 | 1 | 66 | 7 | 2 | 1 | 10 | 3 | 2 | 3 | 6 | 1 | 1 |

Table 2.2 Human *S. aureus* isolates

| Year | MSSA | MRSA |
|---|---|---|
| 2000 | 3 | 2 |
| 2001 | 1 | 3 |
| 2002 | 4 | 1 |
| 2003 | 2 | 1 |
| 2004 | 3 | 1 |
| 2005 | 1 | 6 |
| Total | 14 | 14 |

### 2.2.2 Disc diffusion susceptibility testing

Disc diffusion susceptibility testing using the following antimicrobials; amikacin (30μg), chloramphenicol (10μg), ciprofloxacin (1μg), clindamycin (2μg), erythromycin (5μg), fusidic acid (10μg), gentamicin (10μg), kanamycin (30μg), linezolid (10μg), mupirocin (5μg), oxacillin (1μg), penicillin (1 unit), quinupristin/dalfopristin (15μg), rifampicin (2μg), streptomycin (10μg), sulphamethoxazole (25μg), teicoplanin (30μg), tetracycline (10μg), tobramycin (10μg), trimethoprim (5μg), tylosin (30μg) and vancomycin (5μg) (Oxoid, Basingstoke, UK) was performed following the recommendations of The British Society for Antimicrobial Chemotherapy (BSAC)[20].

Four morphologically similar colonies were touched, with a sterile loop, and transferred into 2ml of sterile distilled water (Biomerieux, France). The organism suspension was adjusted to McFarland 0.5 turbidity standard (1- 2 x $10^8$ colony forming units (cfu/ml) using sterile distilled water and a working solution produced by performing a 1:10 dilution (1 x $10^7$ cfu/ml). Isosensitest agar plates (ISA) (Oxoid, UK) were inoculated, allowed to dry prior to the application of antibiotic discs and incubated at 35-37°C in air for 18-20 hours. For oxacillin susceptibility testing ISA supplemented with 2% NaCl was used and incubated at 30°C in air for 24 hours. After incubation the zones of

inhibition were measured and susceptibility interpretation made based on BSAC criteria.

### 2.2.3 Phage typing

The standard phage typing procedure, as described by Blair and Williams[42], was used with the 23 phages of the international phage set (IPS) for typing human strains of *S. aureus* and four local experimental phages 88A, 90, 83C, and 932 at 100 times the routine test dilution (100X RTD). A 5ml nutrient broth was inoculated with 3-4 colonies of test isolate and incubated for 24 hours at 37°C. Following incubation the broth was poured onto separate Brain Heart Infusion Agar (BHIA) (Oxoid, UK) plates containing 0.04M $CaCl_2$. Excess broth was removed and plates dried prior to spotting with 10µl of bacteriophage. Plates were incubated for 24 hours at 30°C. Phage patterns were interpreted according to established criteria (Table 2.3). All phage typing was performed at the Health Protection Agency, Staphylococcal Reference Laboratory, Collindale, London. Phage typing data was classified into phage clusters and phage types by lytic group and individual lytic pattern respectively.

Table 2.3 Phage typing interpretation criteria

| Plaque number | Recorded value |
|---|---|
| <5 | 1 |
| 6-19 | 2 |
| 20-49 | 3 |
| >50 | 8 |
| Confluent lysis | 9 |
| Inhibition reaction | 0 |

### 2.2.4 PFGE Typing

Genetic relatedness and characterization of the isolates using PFGE typing of *Sma*I (Gibco, UK) digested DNA was carried out by a modification of the protocol previously published by Tenover *et al.* (1995). A colony was inoculated in a Brain Heart Infusion broth (BHIB) and incubated overnight at 37°C without agitation, centrifuged at 12000rpm, and the pellet washed in 0.8 ml NET buffer (10 mM Tris, 1 mM EDTA, 10 mM NaCl), mixed with 20µl of achromopeptidase (10units/µl) (Sigma, UK), and an equal volume of 2% SeaPlague agarose (Flowgen, UK). The cell/agarose suspension was loaded into block moulds (BioRad, UK) and allowed to solidify at 4°C. Cells were lysed by incubation at 50°C for 3 hours in lysis buffer (6 mM Trizma base, 100 mM EDTA, 1 M NaCl, 0.5% Brij 58, 0.2% sodium deoxycholate, 0.5% lauroyl sarcosine). The blocks were washed three times for 10 minutes in TE buffer (10 mM Trizma base, 1 mM EDTA) at room temperature. One quarter of each block was digested with 30 units of *Sma*I for 3 hours according to the manufacturer's instructions and loaded into the wells of 1% PFGE certified agarose gel (BioRad, UK). Electrophoresis was performed in 0.5 x TBE buffer (pH 8) (Invitrogen, UK) by the contour-clamped homogenous electric field method with a CHEF system (BioRad). The fragments were separated with a linear ramped pulse time of 6.8 seconds – 63.8 seconds over a period of 23 hours at 14°C. Gels were stained with 1 µg/mL ethidium bromide (Sigma, UK) solution for 30 minutes, visualised using UV transillumination and digitally photographed. PFGE pattern analysis was performed visually using the Tenover criteria[424] using GelCompar software (Applied Maths). Dendograms were calculated by the unweighted pair group method by using arithmetic averages (UPGMA). A similarity cutoff value of 70% was used to define a cluster[414].

### 2.2.5 PCR amplification of the 16S-23S intergenic spacer region (RS-PCR)

#### 2.2.5.1 DNA extraction

Genomic DNA from *S. aureus* strains was extracted following the method of Kobayashi *et al*[238] and Leonard *et al*[257]. Three to five colonies were suspended in NET buffer (10 mM Tris, 1 mM EDTA, 10 mM NaCl) containing 10 units of achromopeptidase (Sigma, UK) and incubated at 50°C for 10-15 minutes.

#### 2.2.5.2 RS-PCR

Molecular typing of *Staphylococcus aureus* using RS-PCR was performed as described by Kumari *et al*[243], Couto *et al*[83] and Lee & Park[253] using the primers previously published by Jensen *et al*[205]. Two microlitres of extracted DNA was added to 12.5 µl of Reddy Mix PCR Master mix (abgene, UK) (0.2 mM of dATP, dCTP, dGTP, dTTP, 3 mM $MgCl_2$, 0.625 units Taq polymerase, 20 mM Trizma base, 50 mM KCl) and 25 pmol of primers G1 5′ GAA GTC GTA ACA AGG 3′ and L1 5′ CAA GGC ATC CAC CGT 3′. Tissue culture grade water (Sigma, UK) was added to give a final volume of 25 µl. Cycling conditions consisted of 34 cycles of denaturation at 95°C, annealing at 55°C, and extension at 72°C for 1 minute each, followed by a final extension cycle of 72°C for 7 minutes. Six microlitres of PCR product was loaded into 1.5% Mastgel GP agarose (Mast Diagnostics). Electrophoresis was performed in 0.5 x TBE buffer (pH 8) at 100 volts for 10.5 hours. The patterns of the PCR products were compared with the NCTC reference strain 10442.

### 2.2.6 Discriminatory power

The discriminatory power of typing methods was determined using the Simpson index of diversity formula, as shown by Hunter, which reads:

$$D = 1 - \frac{1}{N(N-1)} \sum_{J=1}^{S} nj\,(nj-1)$$

Where D is the index of discriminatory power, $N$ the number of unrelated strains tested, $S$ the number of different types, and $nj$ the number of strains belonging to the $j^{th}$ type, assuming that all strains will be classified into mutually exclusive categories[195].

### 2.2.7 Cophenetic correlation

The cophenetic correlation between isolates was determined using the following formula (Gel Compar™).

$$c = \frac{\Sigma_{i<j}(Y_{ij}-y)(Z_{ij}-z)}{\sqrt{\Sigma_{i<j}(Y_{ij}-y)^2 \Sigma_{i<j}(Z_{ij}-z)^2}}$$

Where $Y_{ij}$ is the distance between objects $i$ and $j$ in Y, $Z_{ij}$ is the cophenetic distance between objects $i$ and $j$, from Z (:,3) and $y$ and $z$ are the average of Y and Z (:,3), respectively.

### 2.2.8 Multilocus sequence typing (MLST)

The nucleotide sequences of internal fragments of seven housekeeping genes were obtained using the primers shown in Table 2.4: carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glp*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), triosephosphate isomerase (*tpi*), and acetyl coenzyme A acetyltransferase (*yqiL*)[122].

Table 2.4 MLST PCR primers

| Gene | Sequence (5'-3') | |
|---|---|---|
| *arcC* | forward | TTGATTCACCAGCGCTATTGTC |
| | reverse | AGGTATCTGCTTCAATCAGCG |
| *aroE* | forward | ATCGGAAATCCTATTTCACATTC |
| | reverse | GGTGTTGTATTAATAACGATATC |
| *glp* | forward | CTAGGAACTGCAATCTTAATCC |
| | reverse | TGGTAAAATCGCATGTCCAATTC |
| *gmk* | forward | ATCGTTTTATCGGGACCATC |
| | reverse | TCATTAACTACAACGTAATCGTA |
| *pta* | forward | GTTAAAATCGTATTACCTGAAGG |
| | reverse | GACCCTTTTGTTGAAAAGCTTAA |
| *tpi* | forward | TCGTTCATTCTGAACGTCGTGAA |
| | reverse | TTTGCACCTTCTAACAATTGTAC |
| *yqiL* | forward | CAGCATACAGGACACCTATTGGC |
| | reverse | CTGTGAGGAATCGATACTGGAAC |

Chromosomal DNA was extracted by the method of Jordens and Pennington[210] modified for use with *S. aureus*. A 1µl loop full of organism was added to 50µl of TE buffer (20mM EDTA, 10mM Tris-HCl) containing 10mM of lysostaphin (Sigma, UK) and heated at 37°C for 15minutes then 95°C for 15minutes. A further 150µl of TE buffer was added, vortexed for 5-10 seconds followed by centrifugation at 4000 rpm for 20 minutes. PCR was carried out in 25µl reactions containing 2µl of extracted DNA, 100nmol of each forward and reverse primer pair, 12µl of Reddy mix PCR master mix (abgene, UK)(0.2 mM of dATP, dCTP, dGTP, dTTP, 3 mM $MgCl_2$, 0.625 units Taq polymerase, 20 mM Trizma base, 50 mM KCl), and 10µl of tissue grade water (Sigma,

UK). PCR was performed in a PTC-200 DNA engine (MJ Research, USA) with an initial 5 minutes denaturation at 95°C, followed by 30 cycles of denaturation at 95°C for 60 seconds, annealing at 55°C for 60 seconds, and extension at 72°C for 60 seconds, with a final extension step of 72°C for 5 minutes then held at 4°C[122].

The PCR products were precipitated with 60µl of 20% polyethylene glycol 8000-2.5 M NaCl solution, vortexed for 5-10 seconds, centrifuged at 1000 rpm for 30 seconds followed by incubation at room temperature for 30 minutes. PCR products were centrifuged at 3000 rpm for 30 minutes at 4°C and the supernatant removed. PCR products were resuspended in 150µl of cold 70% ethanol and centrifuged at 3000 rpm for 30 minutes at 4°C and ethanol removed. The DNA was reprecipitated in 12µl of tissue grade water (Sigma, UK), centrifuged at 1000 rpm for 15 seconds, vortexed for 10-15 seconds three times then stored at -20°C.

Each PCR product was sequence tagged with BigDye fluorescent terminators (BigDye® Terminator v3.1, Applied Biosystems, USA) using the primers from the initial amplification with the addition of 2µl of BigDye. PCR was performed in a PCT-200 DNA engine (MJ Research, Boston, Mass.) with 25 cycles of 96°C for 10 seconds, 50°C for 3 seconds and 60°C for 2 minutes, followed by a reduction in temperature of $0.1°Cs^{-1}$ with a final hold at 4°C. Prior to sequencing each PCR product was purified in 12µl of tissue grade water and 52µl of 1:25 sodium acetate (Sigma, UK):95% ethanol solution, vortexed, centrifuged for 15-20 seconds at 1000 rpm and frozen at -20°C for 30 minutes. Each product was centrifuged at 3500 rpm for 30 minutes and the liquid phase removed. The DNA was further precipitated in 150µl of 70% ethanol, centrifuged at 3500 rpm for 30 minutes, the ethanol removed, and DNA resuspended in tissue grade

water. The elongation products were loaded into a 5% Long-Ranger gel (Cambrex, Rockland, ME) and the sequence of each strand determined with an ABI Prism 3700 DNA sequencer (Applied Biosystems, UK).

The sequences for each locus were compared to the allele sequences in the MLST database (http://www.mlst.net). Alleles without a match in the database were submitted for registration. MLST typing was carried out at the Department of Molecular Epidemiology, Imperial College, London. Sequence types were clustered into groups using eBURST employing the relaxed group definition with five of seven loci (i.e. members of a group differ by a single or two loci).

### 2.2.9 *spa* typing

#### 2.2.9.1 DNA extraction

Chromosomal DNA was extracted following the method previously described in section 2.2.8

#### 2.2.9.2 *spa* gene typing

Amplification of the polymorphic region X of the protein A (*spa*) gene was performed by PCR and the sequence of both strands determined. Two microlitres of extracted DNA was added to 12µl of Reddy mix PCR master mix (abgene, UK)(0.2 mM of dATP, dCTP, dGTP, dTTP, 3 mM $MgCl_2$, 0.625 units Taq polymerase, 20 mM Trizma base, 50 mM KCl) and 100nmol of primers *spa* (F) AGA CGA TCC TTC GGT GA and *spa* (R) CAG CAG TAG TGC CGT TTG. Tissue grade water was added to a final volume of 25µl. The PCRs were performed in a PTC-200 DNA engine (MJ Research,

USA) with an initial denaturation at 95°C for 3 minutes followed by 35 cycles of 95°C for 30 seconds, 50°C for 60 seconds and 72°C for 60 seconds, a final extension at 72°C for 10 minutes then held at 4°C[173].

### 2.2.9.3 *spa* PCR amplified product purification

The amplified products were purified following the method previously described in section 2.2.8

### 2.2.9.4 *spa* amplified product sequence tagging

The amplified products were sequence tagged using the method described in section 2.2.8. and the primers used in section 2.2.9.2.

### 2.2.9.5 *spa* gene sequencing

*spa* gene sequencing was performed following the method in section 2.2.8. The sequences of both *spa* strands were determined using SeqManII ver 5.0 (DNAStar, USA) sequence analysis software. *spa* types as well as BURP clonal complexes (*spa*-CCs) were assigned using the Ridom Staph Type software version 1.4.1 (Ridom GmbH, Wurzburg, Germany). Applying the algorithm BURP, *spa* types were clustered into different groups with the calculated cost between members of a group less than or equal to eight. The calculated cost describes the distance between two *spa* types which is calculated with a pairwise alignment between two *spa* types, and takes into account possible duplications, deletions and mutations of *spa* repeats. *spa* types shorter than five repeats were excluded from analysis because no reliable deduction about ancestries can be made from these types. *spa* types were submitted to the Ridom *spa* server database (http://spa.ridom.de/spatypes.shtml) to determine possible associations with MLST. All

*spa* typing was carried out at the Department of Molecular Epidemiology, Imperial Colllege, London

### 2.2.10 *agr* PCR

Amplification of the *agr* genes I-IV was performed by multiplex-PCR (m-PCR) following the protocol of Gilot et al[156] using the primers *agr*I, *agr*II, *agr*III, *agr*IV and pan, controls, and cycling conditions listed in Table 2.5. m-PCR was performed in a 23µl mixture containing 2µl of template DNA, 2µl primer mix (25pmol) (MWG biotech, Germany), 12.5 µl of Reddy Mix PCR Master mix (0.2 mM of dATP, dCTP, dGTP, dTTP, 3 mM $MgCl_2$, 0.625 units Taq polymerase, 20 mM Trizma base, 50 mM KCl) (ABgene, UK) and 6.5µl tissue grade water (Sigma, UK) in a Techne Genius thermal cycler. PCR products were separated by gel electrophoresis using six microlitres of PCR product loaded in a 1.5% Neuseive agarose (Cambrex, UK) gel in a 0.5% TBE solution (Gibco, UK) at 140V for 90 minutes. Following electrophoresis all gels were stained with 1µg/mL ethidium bromide (Sigma, UK) solution for 30 minutes visualised by uv transillumination (UVP, UK) and the image stored digitally using VisionWorks 32 software. The size of each PCR product was determined by comparison with the control strains and a 100-bp molecular weight ladder (Gibco, UK).

Table 2.5 *agr* primers and PCR conditions

| Primer | Product (bp) | Primer sequence 5'-3' | Positive control strain | PCR conditions |
|---|---|---|---|---|
| *agr* 1 | 440 | GTCACAAGTACTATAAGCTGCGAT | 00.9521.M (ST22) | Initial denature 94°C 5 minutes, 26 cycles of 94°C 30 seconds, 55°C 30 seconds, and 72°C 1 minute, final extension 72°C 10 minutes |
| *agr* 2 | 575 | TATTACTAATTGAAAAGTGGCCATAGC | 97.1948.S (ST5) | |
| *agr* 3 | 323 | GTAATGTAATAGCTTGTATAATAATACCCAG | 00.9523.R (ST30) | |
| *agr* 4 | 659 | CGATAATGCCGTAATACCCG | etb | |
| pan | | ATGCACATGGTGCACATGC | | |

## 2.3 Results

### 2.3.1 Antibiotic resistance phenotypes

Antibiotic susceptibility testing of 233 *S. aureus* isolated from animals using a panel of 22 antibiotics (section 2.2.2) distinguished 32 different resistance phenotypes with a discriminatory value (D) of 88.2% (Table 2.6). Thirty isolates were susceptible to all antibiotics tested. Eight antibiotics (fusidic acid, kanamycin, linezolid, mupirocin, oxacillin, quinupristin/dalfopristin, teicoplanin, and vancomycin) did not contribute to characterization as all isolates were uniformly susceptible. Fourteen resistance phenotypes contained only a single isolate (Table 2.7). The remaining 17 phenotypes contained between two and 53 isolates. Five resistance phenotypes (RP), RP3, RP16, RP19, RP22 and RP24, accounted for 71.4% of resistant isolates with RP16 the most common (53 isolates).

Table 2.6 Diversity indices for phage typing, resistance phenotyping, RS-PCR, PFGE MLST and *spa* typing data

| Typing method | No. of types found | Discriminatory power (D) |
|---|---|---|
| Resistance phenotype | 32 | 88.2% |
| Phage typing | 46 | 83.5% |
| RS-PCR (≥1 band difference) | 27 | 86.2% |
| PFGE (≥1 band difference) | 74 | 93.1% |
| RS-PCR and PFGE | 84 | 93.6% |
| MLST | 18 | 91.4% |
| *spa* typing | 25 | 95.4% |
| MLST and *spa* | 43 | 96.7% |

Table 2.7 Antibiotic resistance phenotypes

| Resistance phenotype (RP) | Antibiogram | No of isolates (%) |
|---|---|---|
| 1 | AK | 1 (0.4) |
| 2 | Cip | 4 (1.7) |
| 3 | Cip,P | 12 (5.2) |
| 4 | Cip,S,P | 2 (0.9) |
| 5 | Da,S,P | 1 (0.4) |
| 6 | Da,T | 1 (0.4) |
| 7 | Da,T,Tob,P | 1 (0.4) |
| 8 | Da,Ty,P | 1 (0.4) |
| 9 | E,Da,T,Cip,Ty | 1 (0.4) |
| 10 | E,Da,T,Cip,Ty,P | 1 (0.4) |
| 11 | E,Da,T,S,Ty,P | 5 (2.1) |
| 12 | E,Da,T,S,Ty,W,P | 1 (0.4) |
| 13 | E,Da,T,Ty | 1 (0.4) |
| 14 | E,Da,T,Ty,P | 6 (2.6) |
| 15 | E,Da,Ty,P | 1 (0.4) |
| 16 | P | 53 (22.8) |
| 17 | Rd,P | 1 (0.4) |
| 18 | Rl | 2 (0.9) |
| 19 | Rl,P | 43 (18.4) |
| 20 | S | 2 (0.9) |
| 21 | S,P | 5 (2.1) |
| 22 | T | 17 (7.3) |
| 23 | T,Ch | 2 (0.9) |
| 24 | T,Cip | 20 (8.6) |
| 25 | T,Cip,P | 2 (0.9) |
| 26 | T,Cip,S,P | 4 (1.7) |
| 27 | T,P | 6 (2.6) |
| 28 | T,S,P | 4 (1.7) |
| 29 | T,S,W,P | 1 (0.4) |
| 30 | T,W,P | 1 (0.4) |
| 31 | Ty | 1 (0.4) |
| 32 | Fully susceptible | 30 (12.9) |

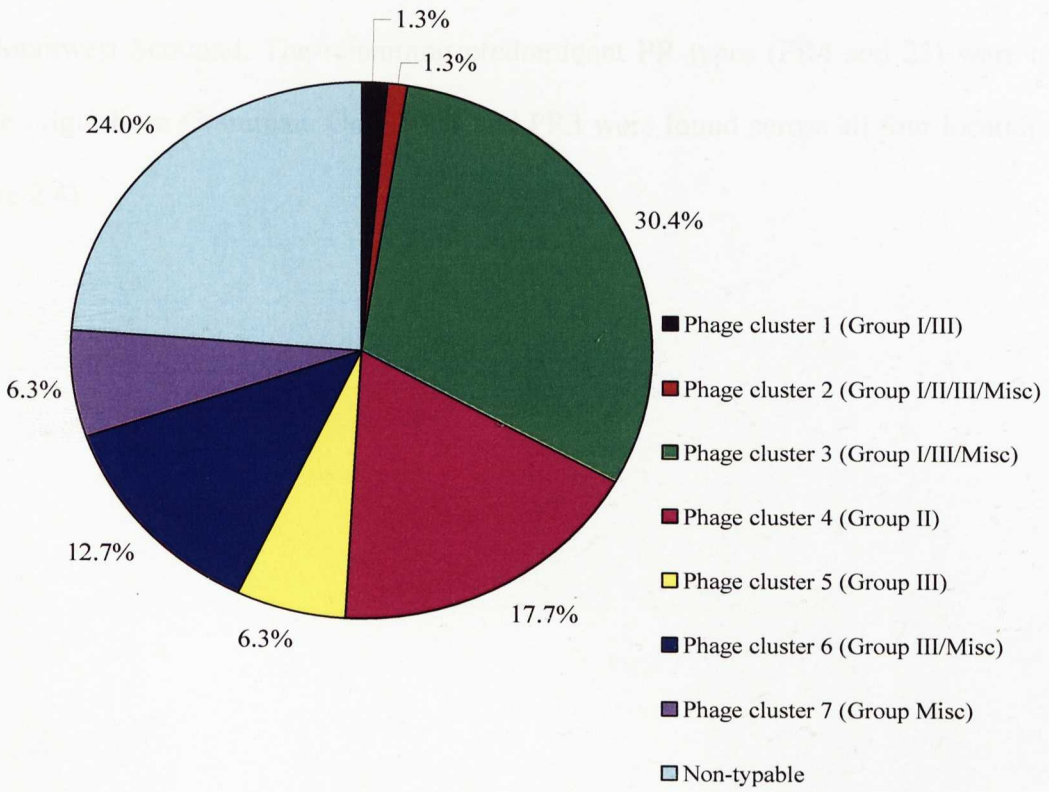
AK, amikacin, Ch, chloramphenicol, Cip, ciprofloxacin, Da, clindamycin, E, erythromycin, P, penicillin, Rd, rifampicin, S, streptomycin, Rl, sulphamethoxazole, Tob, tobramycin, W, trimethoprim, Ty, tylosin

### 2.3.2 Bacteriophage typing

Bacteriophage typing was performed on 79 of the 233 (34%) isolates. Nineteen (25.3%) isolates were non-typable, 16 isolates failed to lyse with any of the phages and three isolates were lytic. All isolates were classified into seven phage clusters, comprising 46 different phage types and a D value of 83.5%. Five phage clusters (representing 52% of isolates) contained isolates susceptible to phages of two or more lytic groups (Figure

2.1). All isolates within phage cluster four (group II phages) were of bovine origin. Isolates within phage cluster five (group III) were from a mixture of animal species, including chicken, milk and pig.

Figure 2.1 Phage typing clusters



### 2.3.3 16S-23S intergenic spacer region PCR (RS-PCR)

Amplification of the 16S-23S intergenic spacer region identified 27 RS-PCR (PR) types (Figure 2.2). The discriminatory power of this method was 86.2% which was lower but not appreciably different to resistance phenotyping (Table 2.6). Thirteen PR types (48%) contained only a single isolate. Five PR types (PR1, 3, 4, 7, and 23) accounted for 81.9% of isolates (Table 2.8 and Figure 2.3). The PR1 type was comprised of 48 isolates. Eighty one percent of which were of avian origin from Highland, Northern Ireland and Southwest Scotland regions. The PR3 and PR7 types contained 60 (25.8%) isolates. The majority of these isolates (81.6%) were from milk samples from Grampian and Southwest Scotland. The remaining predominant PR types (PR4 and 23) were of bovine origin from Grampian. Only PR 1 and PR3 were found across all four locations (Figure 2.4).

Figure 2.2 PR types of animal *S. aureus*

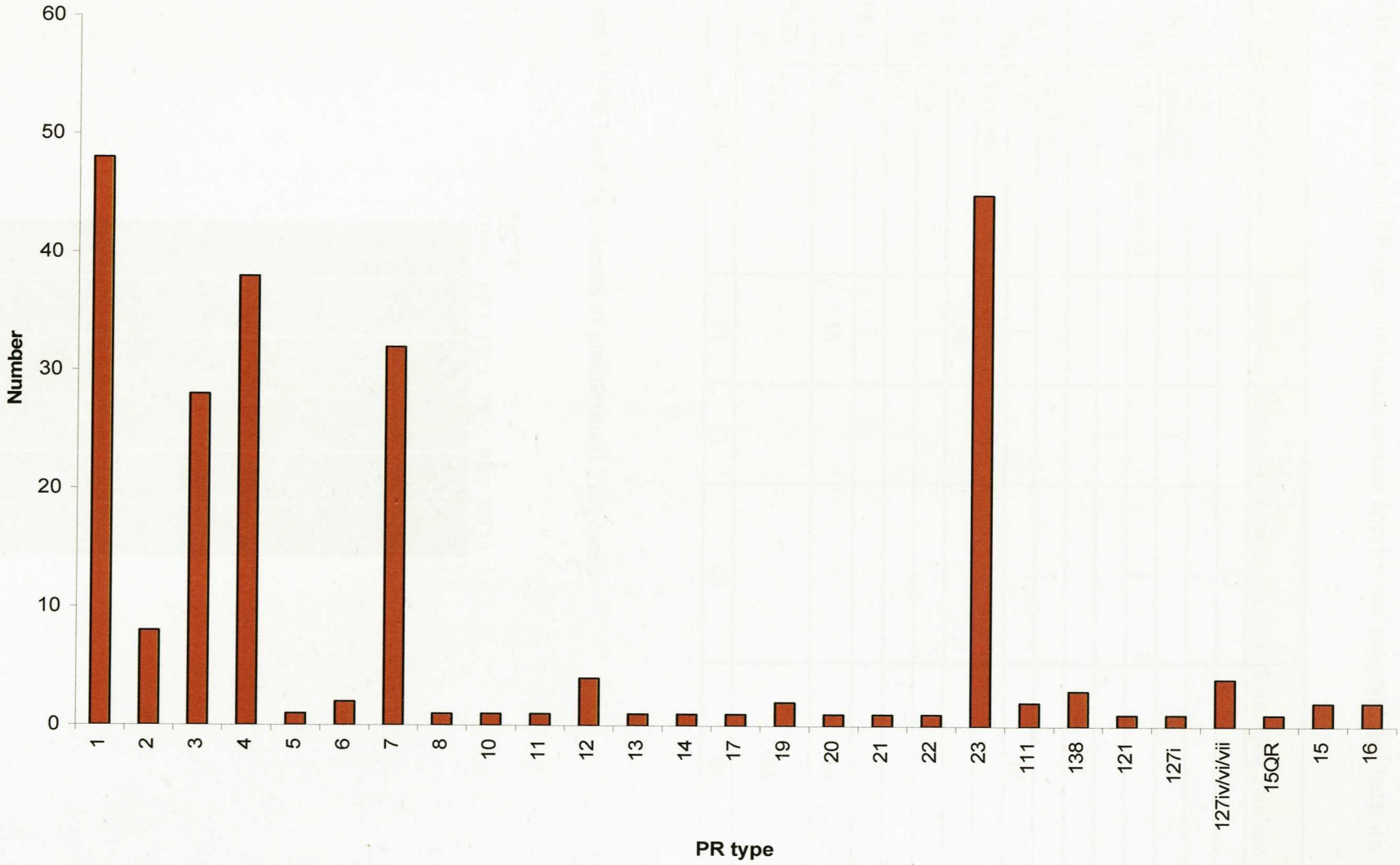
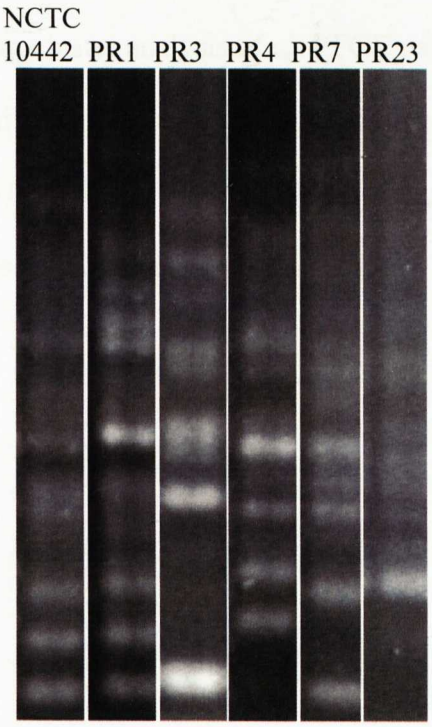


Table 2.8 Predominant PR types including animal species and geographical location

| PR type | Animal species | Grampian | Highland | Northern Ireland | Southwest Scotland |
|---|---|---|---|---|---|
| PR1 (n = 48) | Chicken | | | **33** | 1 |
| | Cow | 8 | | 1 | |
| | Partridge | | 1 | | |
| | Phillips goose hawk | | | 1 | |
| | Pig | | 1 | | |
| | Turkey | | | | 2 |
| PR3 (n = 28) | Chicken | | | 4 | |
| | Milk | 1 | | | **18** |
| | Pheasant | | 5 | | |
| PR4 (n = 38) | Cow | **30** | | | |
| | Milk | 3 | | | |
| | Pig | | 4 | | |
| PR7 (n = 32) | Cow | 2 | | | |
| | Milk | **15** | | 1 | **14** |
| PR23 (n = 45) | Cow | | | | **45** |
| Total | | 59 | 11 | 40 | 80 |

Figure 2.3 PR banding patterns of predominant PR types



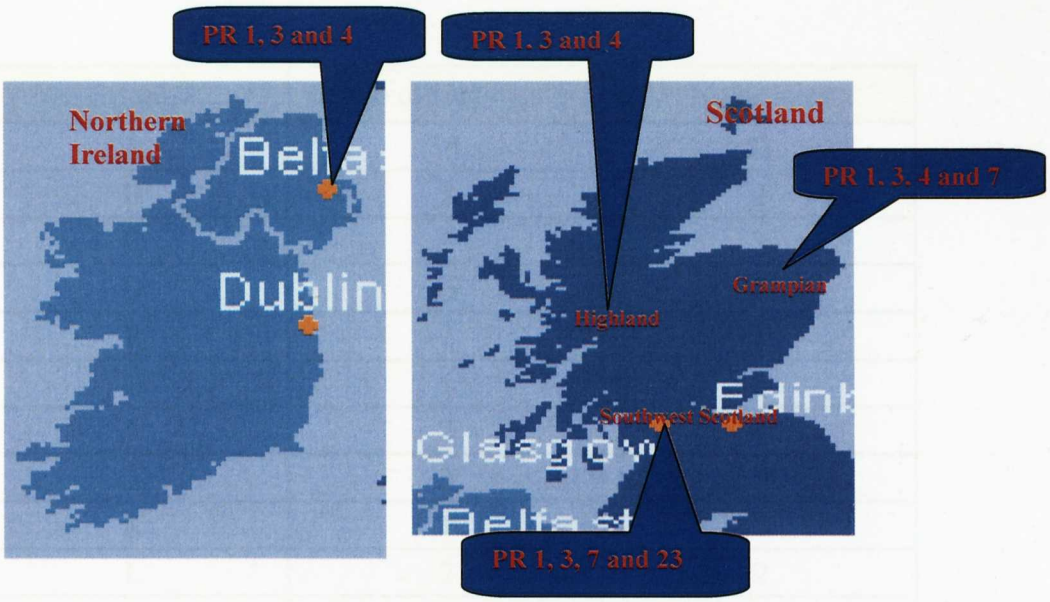



Figure 2.4 Geographical distributions of the predominant PR types

### 2.3.4 Pulsed field gel electrophoresis (PFGE)

Based on visual inspection 74 PF types were recognized among the 233 *S. aureus* isolated from animals. This gave a discriminatory value (D) of 93.1% (Table 2.6). To confirm visual grouping cluster analysis of all PFGE patterns, was performed using Gel compar™ software and resulted in a dendogram comprising 20 clusters using a 70% similarity cut off level (Table 2.9). Eighty four percent of these isolates were distributed in only four PF clusters (A, D, M and T) (Figure 2.5).

Table 2.9 PF groups and subtypes of 233 animal *S. aureus* isolates

| PF group* | No of subtypes | No isolates |
|---|---|---|
| A | 14 | 53 |
| B | 2 | 2 |
| C | 1 | 3 |
| D | 19 | 87 |
| F | 1 | 1 |
| G | 1 | 3 |
| I | 2 | 4 |
| J | 2 | 3 |
| K | 1 | 2 |
| L | 2 | 2 |
| M | 6 | 9 |
| N | 1 | 2 |
| O | 2 | 2 |
| P | 2 | 3 |
| Q | 2 | 4 |
| R | 1 | 1 |
| S | 1 | 1 |
| T | 9 | 42 |
| U | 2 | 6 |
| Y | 3 | 3 |
| Total | 74 | 233 |

* PF groups at a 70% similarity cut off value

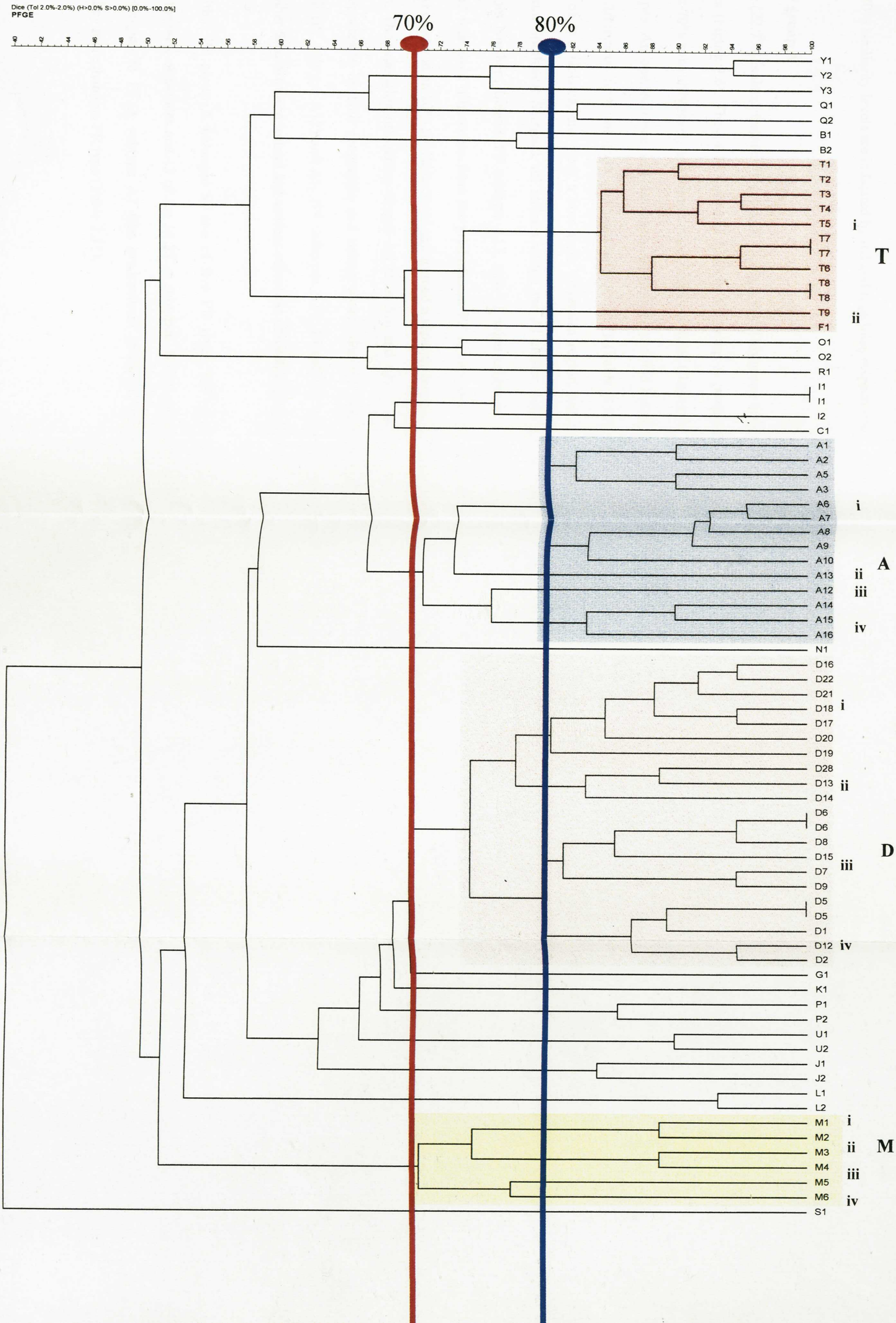

Dice (Tol 2.0%-2.0%) (H>0.0% S>0.0%) [0.0%-100.0%]
PFGE
70%
80%
40 42 44 46 48 50 52 54 56 58 60 62 64 66 68 70 72 74 76 78 80 82 84 86 88 90 92 94 96 98 100
Y1
Y2
Y3
Q1
Q2
B1
B2
T1
T2
T3
T4
T5
i
T7
T7
T6
T8
T8
T9
ii
F1
T
O1
O2
R1
I1
I1
I2
C1
A1
A2
A5
A3
A6
i
A7
A8
A9
A10
A13
ii
A12
iii
A14
A15
iv
A16
A
N1
D16
D22
D21
D18
i
D17
D20
D19
D28
D13
ii
D14
D6
D6
D8
D15
iii
D7
D9
D
D5
D5
D1
D12
iv
D2
G1
K1
P1
P2
U1
U2
J1
J2
L1
L2
M1
i
M2
ii
M3
M4
iii
M5
M6
iv
M
S1

Figure 2.5 Dendogram of PFGE patterns of the 233 animal *S. aureus* strains. The four major PF clusters are colour coded and subclusters are indicated Roman numerals. The 70% and 80% similarity levels are indicated by red and blue lines respectively.

### 2.3.4.1 PF group A

Fifty three (22.7%) isolates formed PF group A (Table 2.10). This group included 14 PF A subtypes (Figure 2.6). The 14 PF subtypes forming PF group A produced a single cluster at a 70% similarity level, and formed four clusters at 80%. Cluster Ai contained nine subtypes, Aii one, Aiii one, and Aiv three respectively and had between one and six band differences from the most prevalent subtype (A7) (Table 2.10). With the exception of two isolates, one from a cow from Grampian region and one from a chicken from Southwest Scotland, all isolates within the Ai cluster were from avian sources from Northern Ireland. PF subtype A13, also of avian origin from Northern Ireland, had four band differences from the predominant PF A subtype and seperated from the Ai cluster at an 80% similarity level and formed a separate branch (Aii) on the dendogram. The seperation of clustres Ai and Aii from Aiii and Aiv at between 70% and 80% appears to be both geographic and animal source derived. Isolates within Cluster Aiii (PF subtype A12) and Aiv (PF subtypes A14, 15 and 16) were all from locations other than Northern Ireland and sources other than chicken (Figure 2.7).

Isolates within PF group A belonged to one of five PR types. PR types 1 and 2 accounted for 94% of isolates and 13 of the 14 PF A subtypes. Within this group only isolates belonging to PF A subtype A7 (the predominant group A subtype) were represented by more than one PR type (Table 2.11).

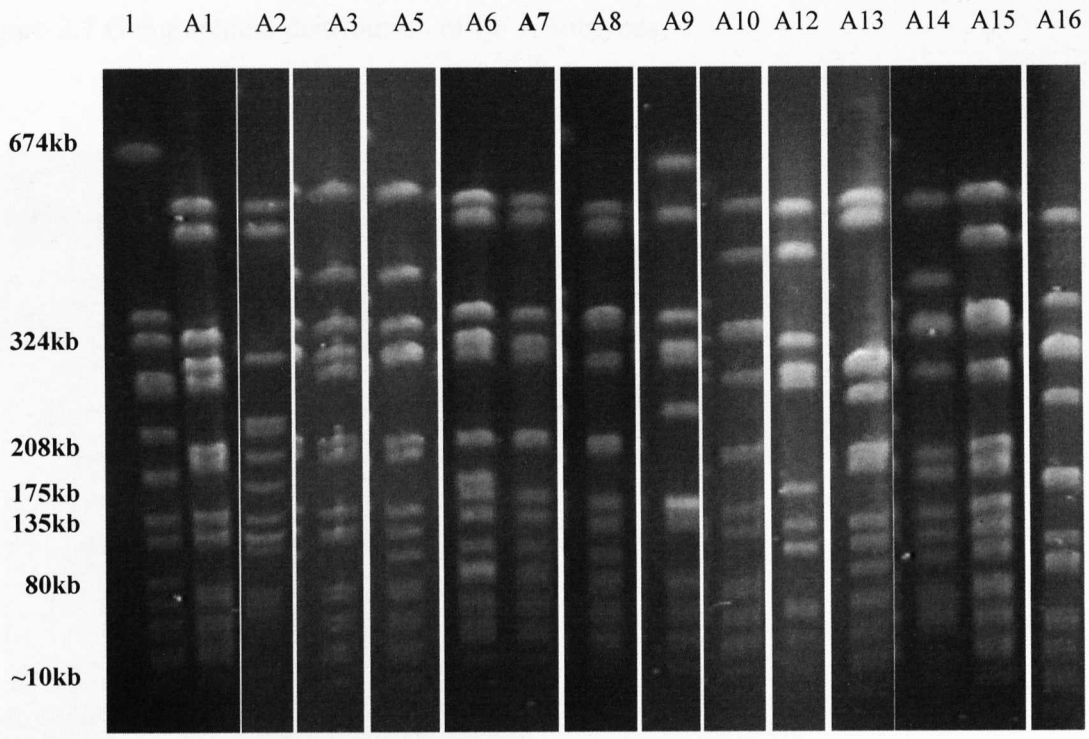
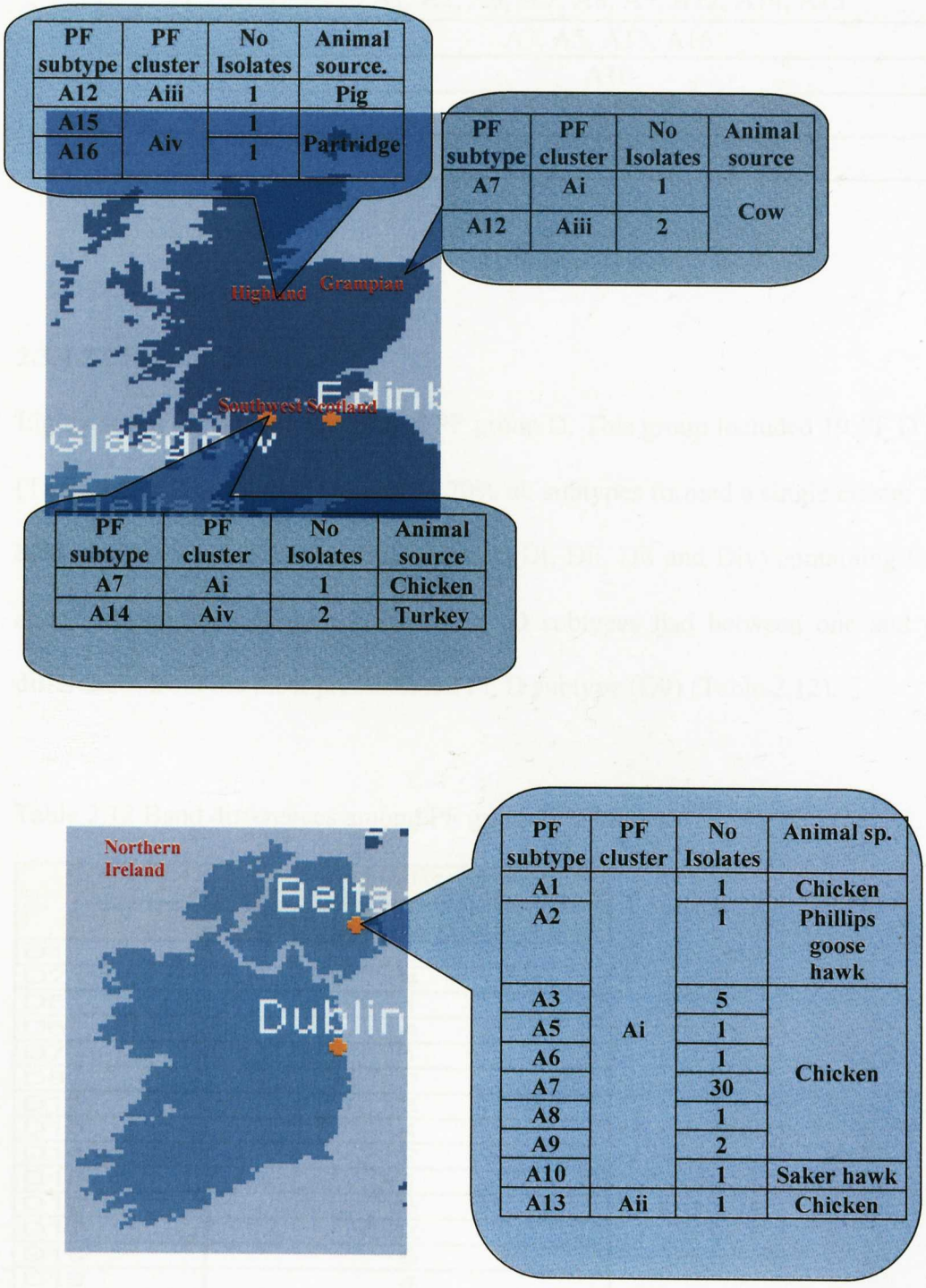



Figure 2.6 PFGE banding patterns representing the 14 PF subtypes forming PF group A. Lane 1 represents the molecular weight standard NCTC 10442.

Table 2.10 Band differences of PF subtypes in PF group A

| PF subtype | Band difference from the predominant PF subtype (A7) |
|---|---|
| A1 | 4 |
| A2 | 6 |
| A3 | 4 |
| A5 | 6 |
| A6 | 1 |
| A8 | 1 |
| A9 | 1 |
| A10 | 3 |
| A12 | 6 |
| A13 | 4 |
| A14 | 6 |
| A15 | 6 |
| A16 | 3 |

Figure 2.7 Geographical distribution of PF A subtypes




| PF subtype | PF cluster | No Isolates | Animal source. |
|---|---|---|---|
| A12 | Aiii | 1 | Pig |
| A15 | Aiv | 1 | Partridge |
| A16 | | 1 | |

| PF subtype | PF cluster | No Isolates | Animal source |
|---|---|---|---|
| A7 | Ai | 1 | Cow |
| A12 | Aiii | 2 | |

| PF subtype | PF cluster | No Isolates | Animal source |
|---|---|---|---|
| A7 | Ai | 1 | Chicken |
| A14 | Aiv | 2 | Turkey |

| PF subtype | PF cluster | No Isolates | Animal sp. |
|---|---|---|---|
| A1 | Ai | 1 | Chicken |
| A2 | | 1 | Phillips goose hawk |
| A3 | | 5 | Chicken |
| A5 | | 1 | |
| A6 | | 1 | |
| A7 | | 30 | |
| A8 | | 1 | |
| A9 | | 2 | |
| A10 | | 1 | Saker hawk |
| A13 | Aii | 1 | Chicken |

Table 2.11 Correlation between PR type and PF group A subtypes

| PR type | PF subtype |
|---|---|
| 1 | A1, A2, A6, **A7**, A8, A9, A12, A14, A15 |
| 2 | A3, A5, A13, A16 |
| 14 | A10 |
| 20 | **A7** |
| 21 | **A7** |

### 2.3.4.2 PF group D

Eighty seven (37%) isolates formed PF group D. This group included 19 PF D subtypes (Table 2.12). At a similarity value of 70% all subtypes formed a single cluster and at an 80% similarity value formed four clusters (Di, Dii, Dii and Div) containing between 3 and 7 PF subtypes (Figure 2.5). All PF D subtypes had between one and six band differences from the most predominant PF D subtype (D9) (Table 2.12).

Table 2.12 Band differences among PF group D subtypes

| PF subtype | Band difference from the predominant PF subtype (D9) |
|---|---|
| D1 | 3 |
| D2 | 4 |
| D5 | 1 |
| D6 | 4 |
| D7 | 1 |
| D8 | 2 |
| D12 | 3 |
| D13 | 4 |
| D14 | 2 |
| D15 | 1 |
| D16 | 6 |
| D17 | 2 |
| D18 | 6 |
| D19 | 4 |
| D20 | 3 |
| D21 | 5 |
| D22 | 5 |
| D28 | 5 |

Isolates within PF group D belonged to one of six PR types (PR3, PR5, PR7, PR8, PR16 and PR23). The PR type 7 contained the largest number of PF D subtypes. The PR types 5 and 8 belonged to singleton PF subtypes D28 and D8 respectively. Four PF D subtypes D6, D9, D12 and D15, representing 68% of the isolates within this group, belonged to more than one PR type. The PR type 3 was the only PR type represented in each of these PF D subtypes (Table 2.13).

Table 2.13 Correlation between PR type and PF group D subtypes

| PR type | PF D Subtypes |
|---|---|
| 3 | D2, D5, **D6, D9, D12**, D14, **D15** |
| 5 | D28 |
| 7 | **D6**, D7, D13, **D15**, D16, D17, D18, D19, D20, D21, D22 |
| 8 | D8 |
| 16 | **D12** |
| 23 | **D9** |

Eighty six of the 87 isolates in PF group D were of bovine origin (cow 47 and milk 39) from the Grampian region (13), Northern Ireland (1) and Southwest Scotland (72). The remaining single isolate (PR5, PF subtype D28) was from a chicken from Northern Ireland. PF subtypes D1 and D28 were exclusive to Northern Ireland. Three PF subtypes (D2, D6 and D13) were common to bovine isolates from Southwest Scotland and Grampian region, three PF subtypes were exclusive to isolates from Grampian region and 11 PF subtypes were exclusive to isolates from Southwest Scotland (Table 2.14).

Table 2.14 Geographic and animal source distribution of PF group D subtypes

| PFG D subtypes | Grampian | | Northern Ireland | | Southwest Scotland | |
|---|---|---|---|---|---|---|
| | Cow | Milk | Chicken | Milk | Cow | Milk |
| D1 | | | | 1 | | |
| D2 | | **1** | | | | **6** |
| D5 | | | | | | 3 |
| D6 | | **3** | | | | **3** |
| D7 | | | | | | 1 |
| D8 | | | | | | 1 |
| D9 | | | | | **45** | **1** |
| D12 | | | | | | 2 |
| D13 | | **2** | | | | **1** |
| D14 | | | | | | 1 |
| D15 | **1** | | | | | **4** |
| D16 | | | | | | 1 |
| D17 | **1** | **3** | | | | |
| D18 | | 1 | | | | |
| D19 | | 1 | | | | |
| D20 | | | | | | 1 |
| D21 | | | | | | 1 |
| D22 | | | | | | 1 |
| D28 | | | 1 | | | |

**Figures in bold are subtypes common to more than one region. Figures in blue are subtypes found in both cattle and milk**

None of the isolates belonging to PF group D were from Highland region. Within PF group D two subclusters, Dii (subtypes D13, 14 and 28) and Div (subtypes D1, 2, 5 and 12), contained PF subtypes from across the remaining three regions (Table 2.14).

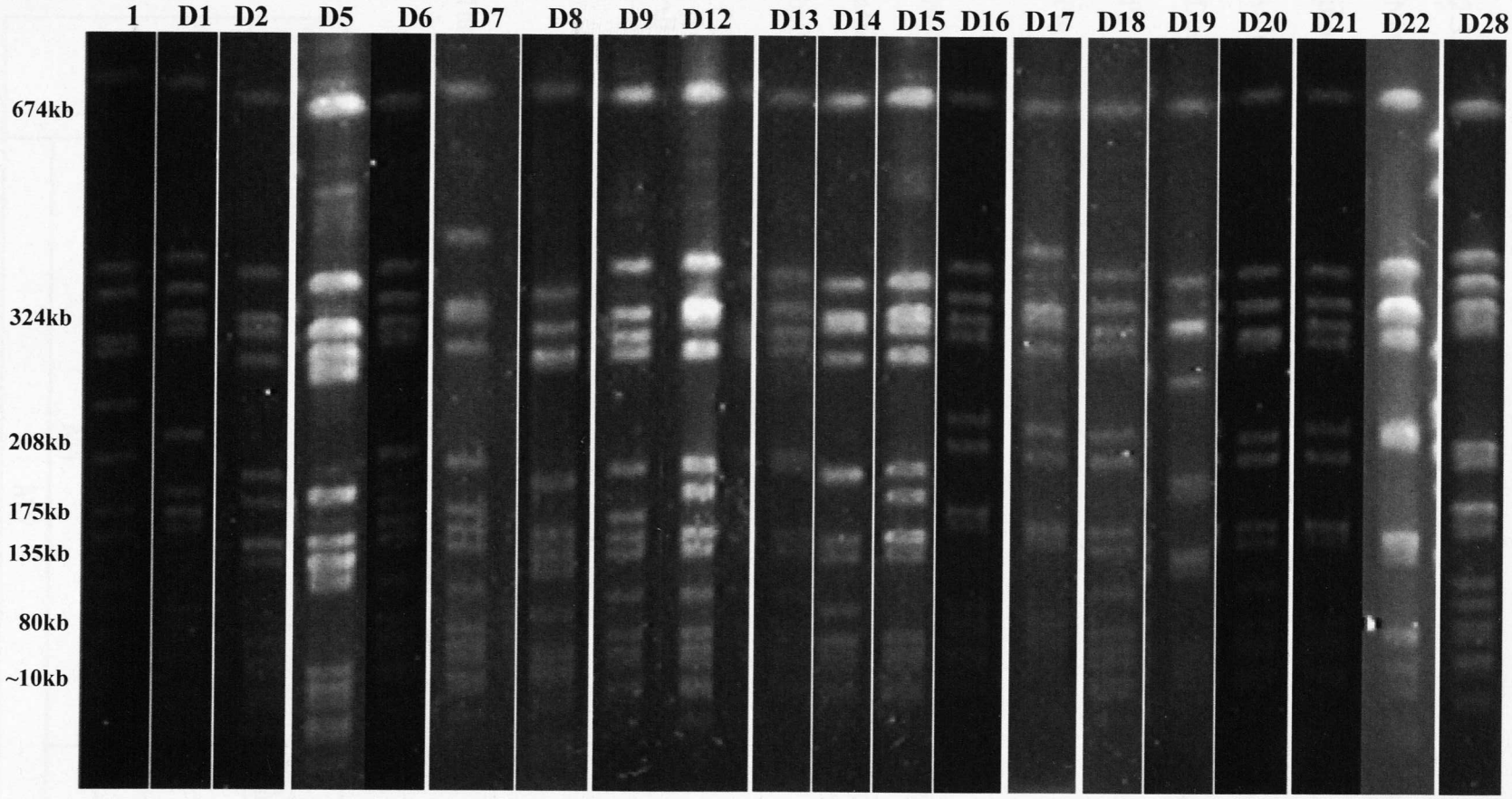



Figure 2.8 PFGE banding patterns representing the 19 PF subtypes forming PF group D. Lane 1 represents the molecular weight standard NCTC 8325.

### 2.3.4.3 PF group M

Nine isolates (4%) formed PF group M. This PF group included six PF subtypes and produced a single cluster at a similarity value of 70% (Figure 2.5). At an 80% similarity value isolates within PF group M produced four PF clusters (Mi, Mii, Miii and Miv). Due to the small number of isolates comprising PF group M there was no predominant PF subtype. All isolates differed from each other by five bands or less (Table 2.15 and Figure 2.9).

PF group M was found only in avian isolates from the Highland region (five pheasant) and Northern Ireland (four chicken). Two PF subtypes (M1 and M5) were common to both locations (Figure 2.10)

All isolates within PF group M belonged to PR type 3.

Table 2.15 Band differences among PF group M subtypes

| PF group M | | Subtype | | | | | |
|---|---|---|---|---|---|---|---|
| | | M1 | M2 | M3 | M4 | M5 | M6 |
| Subtype | M1 | | 1 | 3 | 4 | 1 | 1 |
| | M2 | | | 3 | 4 | 3 | 1 |
| | M3 | | | | 1 | 5 | 5 |
| | M4 | | | | | 4 | 2 |
| | M5 | | | | | | 2 |

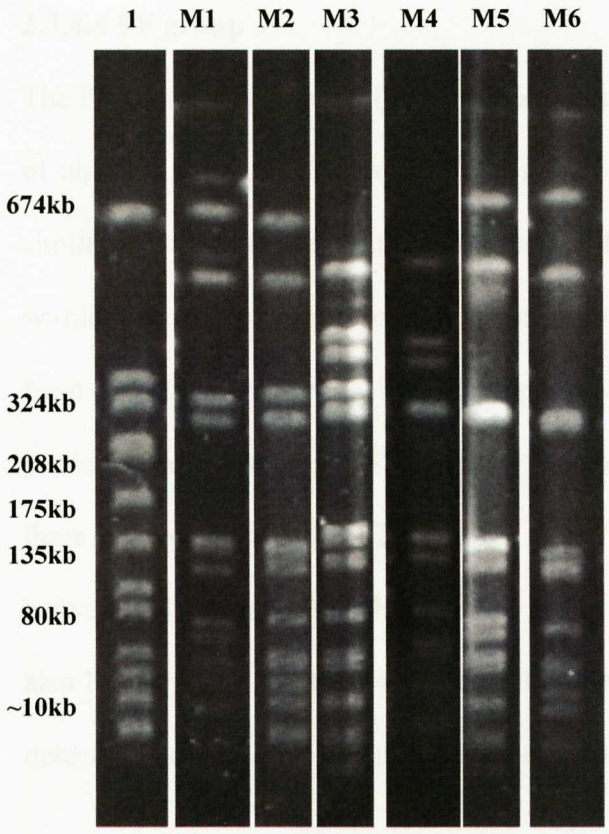
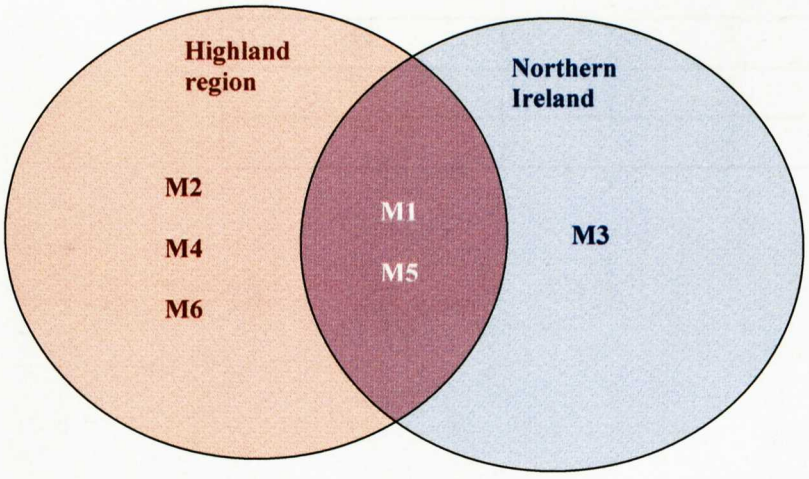



Figure 2.9 PFGE banding patterns representing the 6 PF subtypes forming PF group M. Lane 1 represents a molecular weight standard NCTC 8325.

Figure 2.10 Geographic distributions of PF group M subtypes



### 2.3.4.4 PF group T

The PF group T accounted for 42 (18%) isolates and formed a single cluster, comprised of nine PF subtypes (Figure 2.11), at a 70% similarity value (Figure 2.5). At an 80% similarity value isolates within PF group T formed two clusters (Ti and Tii). Isolates within this group were represented predominantly by bovine sources (cow and milk) from Grampian region. All PF subtypes had five or less band differences from the predominant PF subtype (Table 2.16). Isolates within PF group T belonged to one of three PR types (PR 4, PR 127i and PR 127iv/vi/vii). There was a very close association between PR type and PF subtype in PF group T with 88.1% of isolates within this group also belonging to PR type 4 (Table 2.17). Other than PF group T PR type 4 was only detected in one further PF subtype (PF subtype Y2).

Table 2.16 Band differences between PF T subtypes.

| PF subtype | Band difference from the predominant PF subtype (T8) |
|---|---|
| T1 | 2 |
| T2 | 1 |
| T3 | 5 |
| T4 | 3 |
| T5 | 3 |
| T6 | 5 |
| T7 | 2 |

Table 2.17 Association between PF T subtype and PR type

| PR | PF T subtype | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| 4 | | 1 | 1 | 3 | 5 | 2 | 2 | 21 | 2 |
| 127i | | | | | | | | | 1 |
| 127iv/vi/vii | 1 | | | | | | 3 | | |

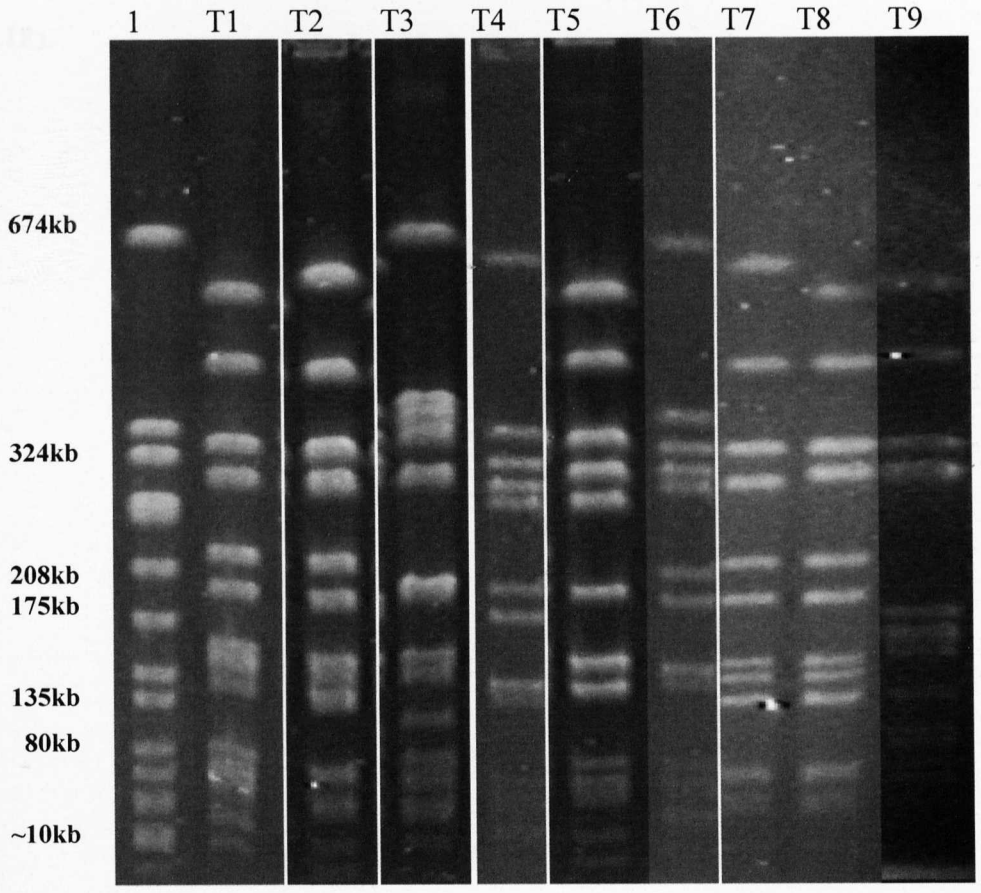



Figure 2.11 PFGE banding patterns representing the 9 PF subtypes forming PF group T. Lane 1 represents a molecular weight standard NCTC 8325.

### 2.3.4.5 Minor PF groups

Nine Pulsed field groups (Figure 2.12) representing 19 distinct PF patterns and containing between two and six isolates were classified as minor PF groups clustering at a similarity cutoff value of 70% (Figure 2.5). Five of these minor PF groups (PF groups J, L, P, Q and U) remained as single clusters at an 80% similarity level. These minor PF groups included isolates from all regions, animal type (companion, livestock and wild), and eight of the 14 animal sources represented in this study (Table 2.18).

Four of the minor PF groups (PF groups B, I, J and Y) belonged to multiple PR types. Isolates belonging to PR types 1 and 7 were found in more than one PF group (Table 2.18).

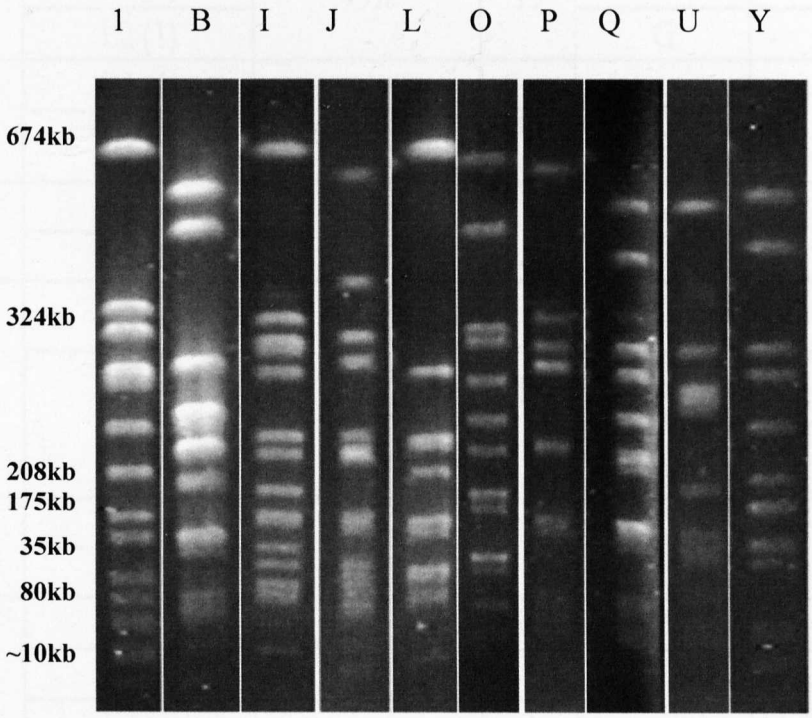



Figure 2.12 Examples of the PFGE banding patterns representing the nine PF groups forming minor clusters. Lane 1 represents a molecular weight standard NCTC 8325. Letters above lanes indicate the minor PF group designation.

Table 2.18 Minor PF groups

| Minor PF groups | PF subtypes (n) | Similarity value | | PR type | Geographic region | Animal source |
|---|---|---|---|---|---|---|
| B | B1 (1) | 78% | | 22 | SWS | Cow |
| | B2 (1) | | | 1 | G | Cow |
| I | I1 (3) | 76% | | 138 | SWS | Horse |
| | | | | | H | Parrot (2) |
| | I2 (1) | | | 13 | NI | Chicken |
| J | J1 (1) | 84% | | 11 | H | Pheasant |
| | J2 (2) | | | 1 | NI | Cow |
| | | | | 17 | | Horse |
| L | L1 (1) | 93% | | 15 | H | Pig |
| | L2 (1) | | | | G | Milk |
| O | O1 (1) | 74% | | 19 | G | Cow |
| | O2 (1) | | | | | |
| P | P1 (1) | 84% | | 7 | G | Milk |
| | P2 (2) | | | | | |
| Q | Q1 (1) | 82% | | 12 | H | Pig |
| | Q2 (3) | | | | G | Milk |
| | | | | | SWS | Lamb |
| U | U1 (4) | 90% | | 7 | SWS | Milk |
| | U2 (2) | | | | | |
| Y | Y1 (1) | 94% | 76% | 15QR | SWS | Milk |
| | Y2 (1) | | | 4 | G | Cow |
| | Y3 (1) | | | 1 | | |

### 2.3.4.6 Singleton PF types

Seven PF patterns representing 12 isolates were classified as singleton PF types (Table 2.19 and Figure 2.13)

Table 2.19 Singleton PF types

| Singleton PF types | PR type | Geographic region | Animal source |
|---|---|---|---|
| C1 (3) | 1 | G | Cow |
| F1 (1) | 121 | G | Milk |
| G1 (3) | 3 and 6 | SWS | Milk |
| K1 (2) | 3 | SWS | Milk |
| N1 (2) | 111 | SWS | Milk |
| | | G | Unknown |
| R1 (1) | 10 | H | Partridge |
| S1 (1) | 16 | SWS | Horse |

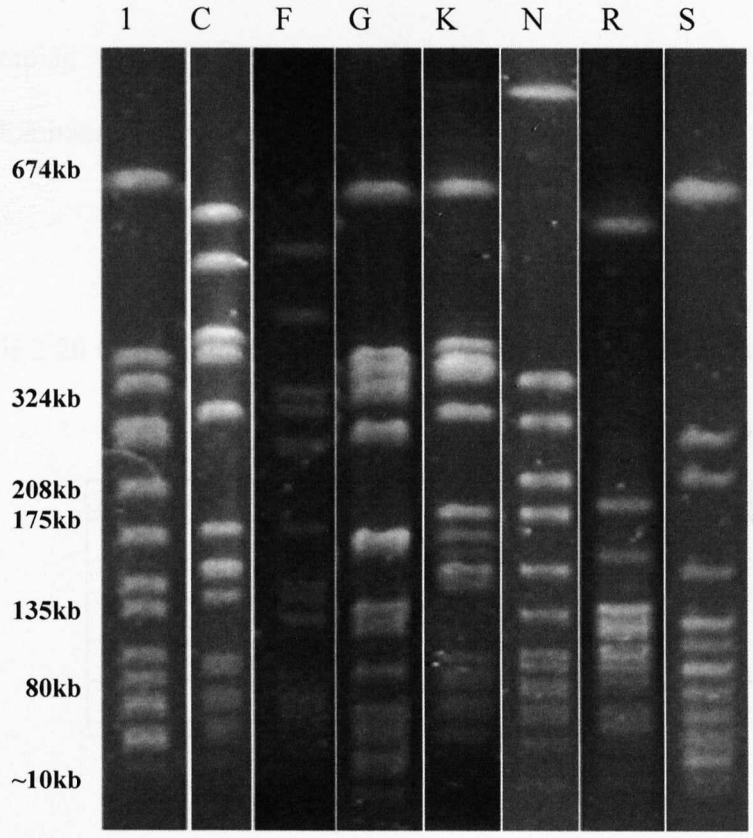



Figure 2.13 PFGE banding patterns representing the seven singleton PF types. Lane 1 represents a molecular weight standard NCTC 8325, Letters above lanes indicate singleton PF pattern

### 2.3.4.7 RS-PCR and PFGE data summary

All *S. aureus* isolated from animals could be type by RS-PCR and PFGE. RS-PCR typing produced 27 PR types, of which five (PR1, PR3, PR4, PR7 and PR23) were predominant, and had a discriminatory power of 86.2%. PFGE typing produced 74 distinct PF banding patterns representing 19 PF groups each with between one and 19 PF subtypes and a discriminatory power of 93.1%. Combining the data of both RS-PCR and PFGE typing did not markedly increase the discriminatory power (Table 2.6). Eighty percent of isolates were distributed amongst four PF groups (PF groups A, D, M and T). PF groups A and M contained isolates predominantly of avian origin from Northern Ireland and Highland region respectively and PF groups D and T contained isolates predominantly of bovine origin predominantly from Southwest Scotland and Grampian region respectively. There was a very close relationship between the predominant PR types and PF groups (Table 2.20).

Table 2.20 Correlation between the predominant RS-PCR types and PFGE groups

| PR type | PF group | Animal source | Geographic region |
|---|---|---|---|
| 1 | A | Avian | NI |
| 3 | D and M | Bovine and Avian | G,H, NI and SWS |
| 4 | T | Bovine | G, H |
| 7 | D | Bovine | G, NI, SWS |
| 23 | D (subtype 9) | Bovine | SWS |

G, Grampian; H, Highland; NI, Northern Ireland; SWS, Southwest Scotland

### 2.3.5 MLST, *spa* and *agr* typing

Further characterization of a subset of 49 *S. aureus* isolates, from the animal isolate collection, was performed. These isolates were selected based on an initial visual analysis of PFGE patterns. Nineteen isolates had PFGE patterns that are commonly found in human clinical *S. aureus* isolated in Scotland. The remaining 30 isolates included a selection of PFGE subtypes from 11 of the 13 animal sources and all four geographic locations. Twenty eight human *S. aureus* isolates were selected for comparison with the animal isolates. These were selected to include PFGE patterns that were indistinguishable to those found amongst the animal isolate collection and a selection of other common human Scottish PFGE types.

#### 2.3.5.1 MLST of animal isolates

A total of 18 different STs were identified among the 49 animal isolates tested (Figure 2.14). The discriminatory power (D) of this method was 91.4% (Table 2.6). Of the 18 STs identified seven (STs 385, 692, 773, 774, 814, 815, and 816) were not found in the MLST database and were classified as novel, of which three also had novel alleles (glpF 108, glpF 109, and aroE 140) (Table 2.21). Four of the STs detected (ST5, ST22, ST30 and ST47) have previously been described within four of the five major MRSA lineages (CC5, CC22, CC30 and CC45).

Figure 2.14 Sequence types found among animal *S. aureus* isolates

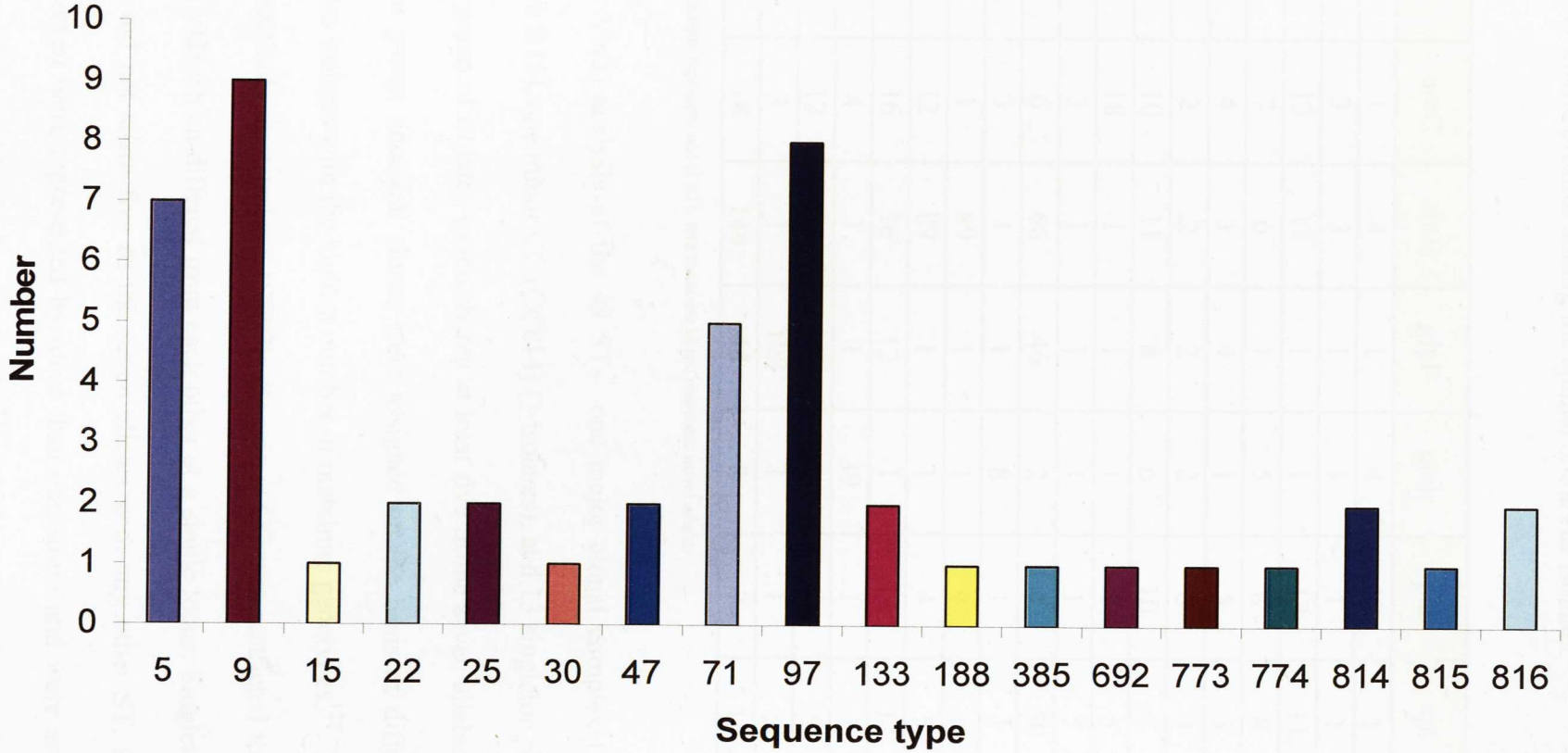


Table 2.21 MLST allelic profiles among the animal *S. aureus* isolates.

| ST | arcC | aroE | glpF | gmk | pta | tpi |
|---|---|---|---|---|---|---|
| 5 | 1 | 4 | 1 | 4 | 12 | 1 |
| 9 | 3 | 3 | 1 | 1 | 1 | 1 |
| 15 | 13 | 13 | 1 | 1 | 12 | 11 |
| 22 | 7 | 6 | 1 | 5 | 8 | 8 |
| 25 | 4 | 1 | 4 | 1 | 5 | 5 |
| 30 | 2 | 2 | 2 | 2 | 6 | 3 |
| 47 | 10 | 11 | 8 | 6 | 10 | 3 |
| 71 | 18 | 1 | 1 | 1 | 1 | 5 |
| 97 | 3 | 1 | 1 | 1 | 1 | 5 |
| 133 | 6 | 66 | 46 | 2 | 7 | 50 |
| 188 | 3 | 1 | 1 | 8 | 1 | 1 |
| **385** | 1 | 89 | 1 | 1 | 4 | 5 |
| **692** | 12 | 89 | **1** | 1 | 4 | 5 |
| **773** | 16 | 16 | 12 | 1 | 13 | 13 |
| **774** | 4 | 1 | 1 | 39 | 1 | 1 |
| **814** | 12 | 89 | **108** | 1 | 4 | 5 |
| **815** | 3 | 1 | **109** | 1 | 1 | 5 |
| **816** | 18 | **140** | 45 | 2 | 7 | 14 |

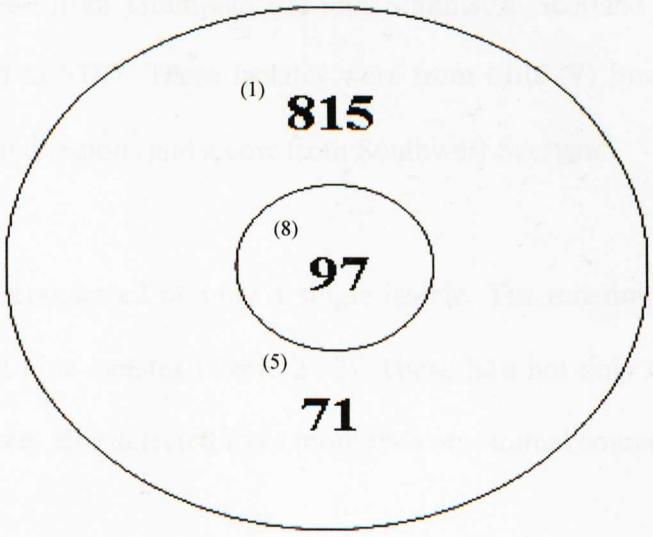
Numbers in purple represent novel STs, and numbers in red represent novel alleles

eBurst (eBurst Ver3) analysis of the 49 STs one major clonal complex (CC97)(14 isolates) (Figure 2.15), one minor CC (CC814) (3 isolates), and 13 singleton STs. A CC is defined as a group of isolates which shared at least five of the seven alleles with one other ST in the group: ancestral clones were assigned on the basis of differing at a single locus from isolates with the highest number of matching genotypes[125]. The major CC had an identifiable ancestral ST (ST97); the minor CC was restricted to two STs (ST814 and ST692) which differed from each other at a single locus; Singleton clones are STs which did not share five of the seven alleles with any other ST. Of the 13 singleton STs seven were represented by more than one strain and were assigned as singleton clones (clones with no clonal variants)[132]. The largest singleton clone in this data set was ST9, which was represented by nine isolates, followed by ST5 represented

by seven isolates, and ST22, ST25, ST47, ST133 and ST816 represented by two strains each.

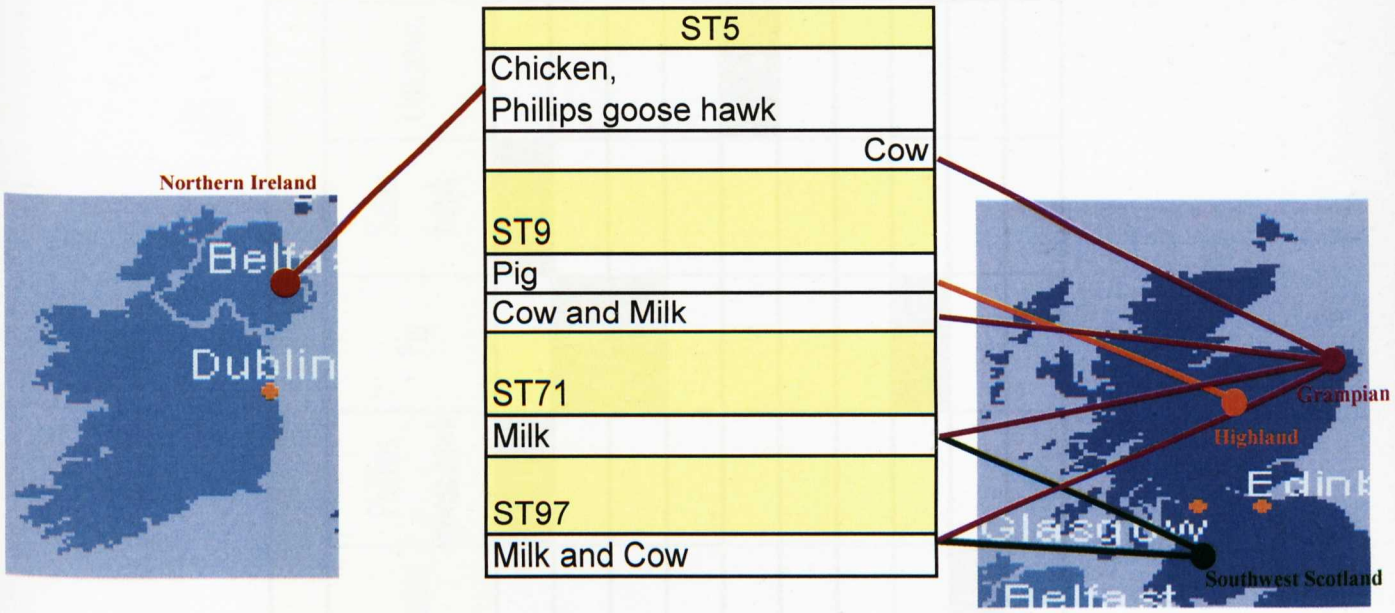
Four STs (ST5, ST9, ST71 and ST97) were predominant and accounted for 60% of all isolates examined. Seven of the 49 isolates tested belonged to ST5. Six of these isolates were from avian sources from Northern Ireland (Figure 2.16).

Figure 2.15 BURST diagram of CC97. The predicted clonal ancestor is in the central ring, SLVs are in the outer ring.




( ) number of isolates within the given ST

Figure 2.16 Distribution of the four predominant STs



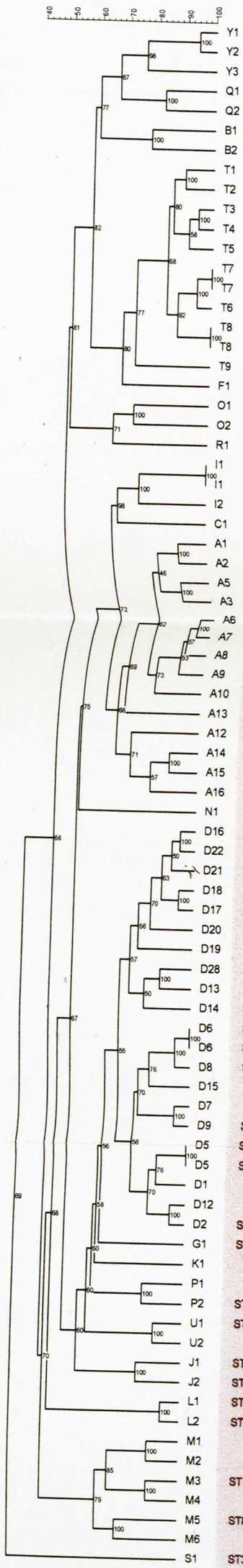

Nine isolates belonged to ST9. These isolates were from cattle (7) from the Grampian region and pigs (2) from the Highland region. The ST71 comprised of five isolates all from milk sources from Grampian (2) and Southwest Scotland regions (3). Eight isolates belonged to ST97. These isolates were from Milk (7) from the Grampian and Southwest Scotland regions and a cow from Southwest Scotland.

Eight STs were represented by only a single isolate. The remaining 10 STs contained between two and nine isolates (Table 2.22). These STs not only contained more than one isolate but were also detected from more than one animal source

Five of the animal species represented in this study belonged to more than one ST, with a maximum of five STs detected among isolates from cattle.

Table 2.22 Distribution of STs containing more than one isolates.

| ST | Animal source | | | | | | | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Chicken | Cow | Horse | Lamb | Milk | Parrot | Pheasant | Phillips goose hawk | Pig | Saker hawk | Unknown | |
| 5 | 4 | 1 | | | | | | 1 | | 1 | | 7 |
| 9 | | 4 | | | 3 | | | | 2 | | | 9 |
| 22 | | | | | 1 | | | | 1 | | | 2 |
| 25 | | | 1 | | | 1 | | | | | | 2 |
| 47 | | 1 | | | | | | | | | 1 | 2 |
| 71 | | | | | 5 | | | | | | | 5 |
| 97 | | 1 | | | 7 | | | | | | | 8 |
| 133 | | | | 1 | | | | | 1 | | | 2 |
| 814 | 1 | | | | | | 1 | | | | | 2 |
| 816 | | 1 | 1 | | | | | | | | | 2 |




| ST | *spa* type Kreiswerth | *spa* type Ridom | | *agr* group | Isolate source animal | Isolate source geographic |
|---|---|---|---|---|---|---|
| | | | | | Milk | Southwest Scotland |
| | | | | | Cow | Grampian |
| | | | | | Cow | Grampian |
| ST133 | D2GFMJEMMJJEMMMMJQ | 03-12-21-17-23-13-17-17-23-13-17-17-17-17-23-24 | t1428 | I | Pig | Highland |
| ST133 | D2GFMJEMMJJEMMMMJQ | 03-12-21-17-23-13-17-17-23-13-17-17-17-17-23-24 | t1428 | I | Lamb | Southwest Scotland |
| | | | | | Cow | Southwest Scotland |
| | | | | | Cow | Grampian |
| ST9 | UKJJAGJAB | 07-16-23-23-02-12-23-02-34 | t337 | II | Milk | Grampian |
| ST9 | UKJJAGJAB | 07-16-23-23-02-12-23-02-34 | st337 | II | Cow | Grampian |
| ST9 | UKJJAGJAB | 07-16-23-23-02-12-23-02-34 | t337 | II | Cow | Grampian |
| ST9 | UKJJAGJAB | 07-16-23-23-02-12-23-02-34 | t337 | II | Cow | Grampian |
| | | | | | Cow | Grampian |
| ST9 | UKJJAGJAB | 07-16-23-23-02-12-23-02-34 | t337 | II | Milk | Grampian |
| ST9 | UJAGJAB | 07-23-02-12-23-02-34 | t1419 | II | Cow | Grampian |
| ST9 | UKJJAGJAB | 07-16-23-23-02-12-23-02-34 | t337 | II | Milk | Grampian |
| | | | | II | Pig | Highland |
| ST15 | UJG-BBGGJAGJ | 07-23-12-34-34-12-12-23-02-12-23 | t084 | | Cow | Grampian |
| | | | | II | Cow | Grampian |
| | | | | | Milk | Grampian |
| ST773 | ZDMJ-new-DMO | 04-20-17-23-117-20-17-25 | t1420 | | Cow | Grampian |
| | | | | IV | Cow | Grampian |
| ST25 | ZFGU2DMGGM | 04-21-12-41-20-17-12-12-17 | t078 | | Partridge | Highland |
| ST25 | ZFGU2DMGGM | 04-21-12-41-20-17-12-12-17 | t1423 | I | Horse | Southwest Scotland |
| | | | | I | Parrot | Highland |
| ST5 | TJMBMDMGMK | 26-23-17-34-17-20-17-12-17-16 | t002 | | Chicken | Northern Ireland |
| ST5 | | | | II | Cow | Grampian |
| ST5 | TJMBMDMGMK | 26-23-17-34-17-20-17-12-17-16 | t002 | II | Chicken | Northern Ireland |
| ST5 | TJMBMDMGMMK | 26-23-17-34-17-20-17-12-17-17-16 | t306 | II | Phillips Goose Hawk | Northern Ireland |
| | | | | II | Chicken | Northern Ireland |
| ST5 | | | | | Chicken | Northern Ireland |
| *ST5* | *TJMBMDMGMK* | *26-23-17-34-17-20-17-12-17-16* | *t002* | II | *Chicken* | *Northern Ireland* |
| *ST5* | | | | *II* | *Chicken* | *Northern Ireland* |
| ST5 | | | | II | Chicken | Northern Ireland |
| ST5 | TJMBMDMGMMK | 26-33-17-34-17-20-17-12-17-17-16 | t306 | II | Chicken | Northern Ireland |
| | | | | II | Saker Hawk | Northern Ireland |
| | | | | | Chicken | Northern Ireland |
| | | | | | Cow | Grampian |
| | | | | | Turkey | Southwest Scotland |
| | | | | | Partridge | Highland |
| | | | | | Partridge | Highland |
| ST47 | XKB | 08-16-34 | t026 | I | Cow | Grampian |
| ST97 | UJGFMBPB | 07-23-12-21-17-34-33-34 | t224 | I | Milk | Southwest Scotland |
| | | | | | Milk | Southwest Scotland |
| ST97 | UJGFMBPB | 07-23-12-21-17-34-33-34 | t224 | I | Milk | Southwest Scotland |
| | | | | | Milk | Grampian |
| ST71 | ZM | 04-17 | t524 | I | Milk | Grampian |
| | | | | | Milk | Southwest Scotland |
| | | | | | Milk | Northern Ireland |
| ST 774 | TJFKBPE | 26-23-21-16-34-33-13 | t177 | III | Chicken | Northern Ireland |
| | | | | | Milk | Grampian |
| ST97 | UJGFMBPB | 07-23-12-21-17-34-33-34 | t224 | I | Milk | Southwest Scotland |
| ST97 | UJGFMBBBPB | 07-23-12-21-17-34-34-34-33-34 | t267 | I | Milk | Grampian |
| ST71 | ZM | 04-17 | t524 | I | Milk | Southwest Scotland |
| ST71 | Z | 04 | t528 | I | Milk | Southwest Scotland |
| | | | | | Milk | Southwest Scotland |
| | | | | | Milk | Southwest Scotland |
| ST97 | UJG(new)BBPB | 07-23-12-118-34-34-33-34 | t1421 | I | Cow | Southwest Scotland |
| ST97 | UJGFMBBBBBPB | 07-23-12-21-17-34-34-34-34-33-34 | t527 | I | Milk | Southwest Scotland |
| ST815 | UJGFMBBBBBPB | 07-23-12-21-17-34-34-34-34-33-34 | t527 | I | Milk | Southwest Scotland |
| | | | | | Milk | Northern Ireland |
| | | | | | Milk | Southwest Scotland |
| ST188 | UJGFMEBBPB | 07-23-12-21-17-13-34-34-33-34 | t1428 | I | Milk | Southwest Scotland |
| ST97 | UJGFMBPB | 07-23-12-21-17-34-33-34 | t224 | I | Milk | Southwest Scotland |
| | | | | | Milk | Southwest Scotland |
| | | | | | Milk | Northern Ireland |
| ST71 | ZM | 04-17 | t524 | I | Milk | Grampian |
| ST71 | ZM | 04-17 | t524 | I | Milk | Southwest Scotland |
| | | | | | Milk | Grampian |
| ST385 | UJGFQPLM | 07-23-12-21-24-33-22-17 | t213 | II | Pheasant | Highland |
| ST816 | WMBBBPB | 15-17-34-34-34-33-34 | t1429 | II | Cow | Northern Ireland |
| ST22 | TJEJCMOMOKR | 26-23-13-23-05-17-25-17-25-16-28 | t223 | I | Pig | Highland |
| ST22 | TJEJCMOMOKR | 26-23-13-23-05-17-25-17-25-16-28 | t223 | I | Milk | Grampian |
| | | | | | Chicken | Northern Ireland |
| | | | | | Pheasant | Highland |
| ST 692 | TJGBFMBOM | 26-23-12-34-21-17-34-25-17 | | I | Chicken | Northern Ireland |
| | | | | | Pheasant | Highland |
| ST814 | TJGBFMBBOM | 26-23-12-34-21-17-34-34-25-17 | t1422 | I | Chicken | Northern Ireland |
| | | | | | Pheasant | Highland |
| ST30 | WGKAKAOMQQ | 15-12-16-02-16-02-25-17-24-24 | t012 | III | Horse | Southwest Scotland |

Figure 2.17 Dendogram of PFGE patterns including MLST, *spa* typing and *agr* typing data of selected isolates.

Cophenetic correlation analysis of each PF subtype was performed (Figure 2.17). The cophenetic correlation for a cluster tree is defined as the linear correlation coefficient between the cophenetic distances obtained from the tree, and the original distances (or dissimilarities) used to construct the tree. Thus, it is a measure of how faithfully the tree represents the dissimilarities among observations. Cophenetic correlation supported the separation of strains into clusters.

#### 2.3.5.2 *spa* typing of animal isolates

*spa* gene sequence analysis, of the 49 animal isolates tested, resulted in the detection of 25 *spa* types varying in length between one (t528) and 16 (t1428) repeats, including 10 types (t1418, t1419, t1420, t1421, t1422, t1423, t1428, t1429, t1430 and NEW) not in the Ridom database. Using the algorithm BURP, Ridom StaphType™ software, *spa* types were grouped together if the calculated cost between members of a group was less than or equal to eight and formed four *spa* clonal complexes (*spa* CC) (*spa* CC267, CC1430, CC78 and CC2) and 10 singleton *spa* types[414]. Since clustering parameters excluded *spa* types shorter than five repeats *spa* types' t026, t524 and t528 were excluded from BURP grouping. The most frequent *spa* types were t337 (seven isolates), t002 (five isolates), and t224 (five isolates) (Table 2.23).

Table 2.23 *spa* types of the 49 animal *S. aureus* isolates

| *spa* type | Kreisworth *spa* type | Ridom *spa* type | No. isolates |
|---|---|---|---|
| t002 | TJMBMDMGMK | 26-23-17-34-17-20-17-12-17-16 | 5 |
| t012 | WGKAKAOMQQ | 15-12-16-02-16-02-25-17-24-24 | 1 |
| t026 | XKB | 08-16-34 | 1 |
| t078 | ZFGU2DMGGM | 04-21-12-41-20-17-12-12-17 | 2 |
| t84 | UJGBBGGJAGJ | 07-23-12-34-34-12-12-23-2-12-23 | 1 |
| t177 | TJFKBPE | 26-23-21-16-34-33-13 | 1 |
| t213 | UJGFQPLM | 07-23-12-21-24-33-22-17 | 1 |
| t223 | TJEJCMOMOKR | 26-23-13-23-05-17-25-17-25-16-28 | 2 |
| t224 | UJGFMBPB | 07-23-12-21-17-34-33-34 | 5 |
| t267 | UJGFMBBBPB | 07-23-12-21-17-34-34-34-33-34 | 1 |
| t306 | TJMBMDMGMMK | 26-23-17-34-17-20-17-12-17-17-16 | 2 |
| t337 | UKJJAGJAB | 07-16-23-23-02-12-23-02-34 | 7 |
| t524 | ZM | 04 17 | 1 |
| t527 | UJGFMBBBBBPB | 07-23-12-21-17-34-34-34-34-34-33-34 | 2 |
| t528 | Z | 4 | 1 |
| t1418 | UJGFMEBBPB | 07-23-12-21-17-13-34-34-33-34 | 1 |
| t1419 | UJAGJAB | 07-23-02-12-23-02-34 | 1 |
| t1420 | ZDMJ(new spa sequence)DMO | 04-20-17-23-117-20-17-25 | 1 |
| t1421 | UJG(new spa sequence)BBPB | 07-23-12-118-34-34-33-34 | 1 |
| t1422 | TJGBFMBBOM | 26-23-12-34-21-17-34-34-25-17 | 2 |
| t1428 | D2GFMJEMMJEMMMMJQ | 03-12-21-17-23-13-17-17-23-13-17-17-17-17-23-24 | 2 |
| t1429 | WMBBBPB | 15-17-34-34-34-33-34 | 2 |
| t1430 | UKJAGJAB | 07-16-23-02-12-23-02-34 | 1 |
| new | TJGBFMBOM | 26-23-12-34-21-17-34-25-17 | 1 |

*spa* CC 267 contained seven *spa* types, including three novel *spa* types (t1418, t1421 and t1429), one novel *spa* repeat (r118) and varied in length between seven (t1429) and 12 (t527) repeats (Figure 2.18). *spa* types within this clonal complex were closely associated with isolates belonging to MLST CC97 and isolates of bovine origin (cow and milk) from southwest Scotland (Table 2.24).

Figure 2.18 BURP analysis of *spa* CC267 (the calculated cost between *spa* types is also included).

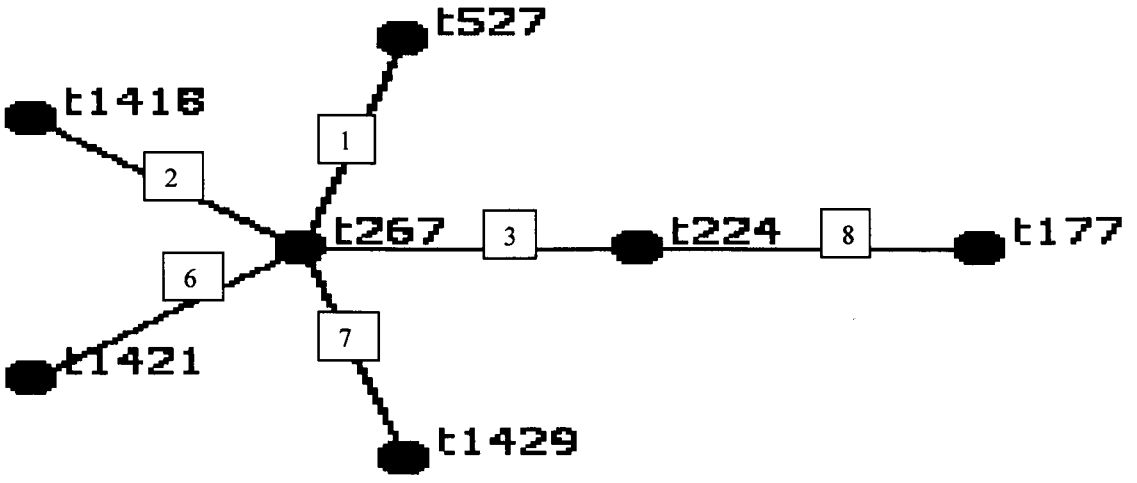
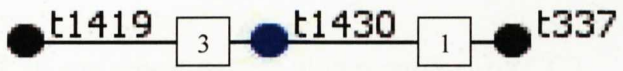


Table 2.24 Association between animal source and geographic region within *spa* CC267

| *spa* type (no isolates) | Animal source | Geographic region |
|---|---|---|
| t224 (1) | Milk | SWS |
| t267 (1) | Milk | G |
| t527 (2) | Milk | SWS |
| t1421 (1) | Cow | SWS |
| t1418 (1) | Milk | SWS |
| t177 (1) | Chicken | NI |
| t1429 (2) | Cow, Horse | NI |

*spa* CC 1430 comprised of three *spa* types, including two novel *spa* types (t1419 and the predicted founder t1430) varying in length between seven (t1419) and nine (t337) repeats. *spa* types within *spa* CC1430 clustered at a cost of less than or equal to three (Figure 2.19). This *spa* CC contained only bovine isolates belonging to MLST CC9.

Figure 2.19 BURP analysis of *spa* CC1430



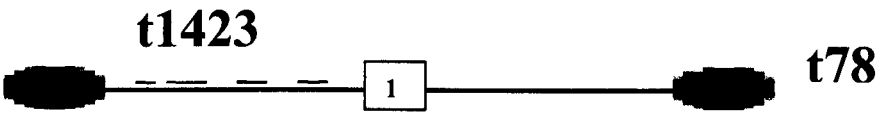

Blue spot indicates the *spa* type of the predicted ancestral strain

*spa* CC78 comprised of two *spa* types, both isolates from companion animals (ST25) from different geographic locations, and included one novel *spa* type (t1423). *spa* type t1423 differed from t78 by the insertion of an additional r12 and r41 in the repeat succession (Table 2.25) and clustered at a calculated cost of less than or equal to one (Figure 2.20)

Table 2.25 *spa* repeat pattern alignment for *spa* CC78

| *spa* type | *spa* repeat pattern allignment | Animal source | Geographic region |
|---|---|---|---|
| t078 | 04-21-12-41 - - 20-17-12-12-17 | Horse | SWS |
| t1423 | 04-21-12-41-12-41-20-17-12-12-17 | Parrot | H |

Figure 2.20 BURP analysis of *spa* CC78

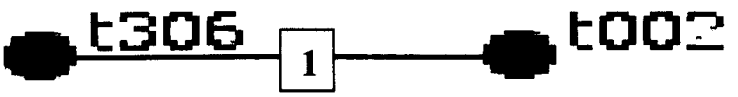



*spa* CC2 comprised of two *spa* types (t002 and t306). *spa* type t306 differed from t002 by the insertion of an additional r17 in the repeat succession (Table 2.26) and clustered at a calculated cost of less than or equal to one (Figure 2.21). All isolates within *spa* CC2 belonged to ST5.

Table 2.26 *spa* repeat pattern alignment for *spa* CC2

| *spa* type | *spa* repeat pattern allignment |
|---|---|
| t002 | 26-23-17-34-17-20-17-12-17 - 16 |
| t306 | 26-23-17-34-17-20-17-12-17-17-16 |

Figure 2.21 BURP analysis of *spa* CC2




*spa* type t1422 and the unassigned new *spa* type differed from each other by a single *spa* repeat (r34). As no type allocation was available for the new *spa* type cluster analysis by BURP was not possible. Although the repeat succession data indicate that they are closely related (Table 2.27).

Table 2.27 *spa* repeat alignment of *spa* type t1422 and a new unassigned *spa* type

| *spa* type | *spa* repeat pattern alignment |
|---|---|
| t1422 | 26-23-12-34-21-17-34-34-25-17 |
| NEW | 26-23-12-34-21-17-34 - 25-17 |

Since clustering parameters excluded *spa* types shorter than five repeats, three *spa* types (t026, t524 and t528) were excluded from BURP analysis. *spa* type t026 (repeat pattern 08-16-34) was represented by two isolates, one cow and one unknown animal source from Grampian and SWS respectively.

Visual inspection of *spa* type's t524 and t528 showed a clear similarity in repeat patterns (Table 2.28). *spa* typing was unable to cluster these isolates within a *spa* CC.

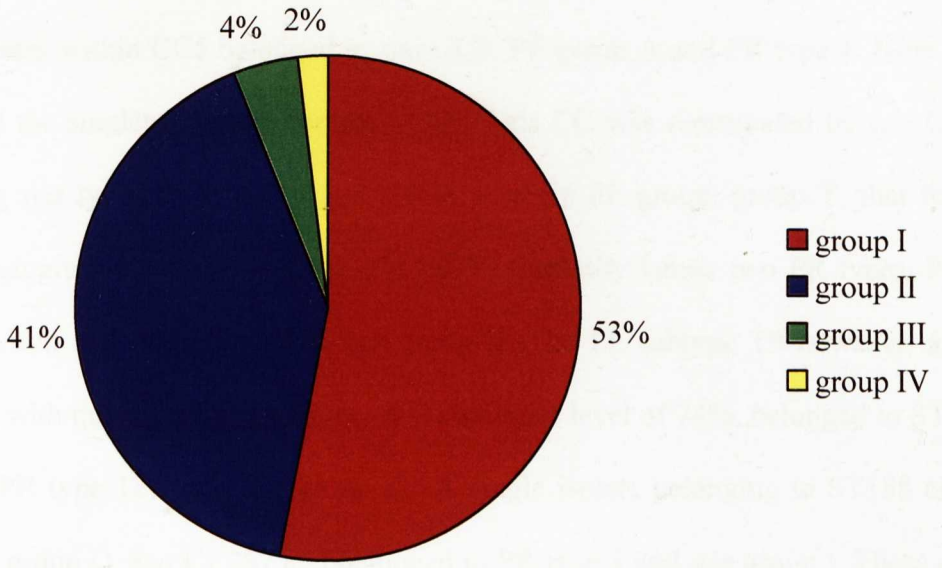
Table 2.28 *spa* repeat alignment of *spa* types t524 and t528

| *spa* type | *spa* repeat pattern alignment |
|---|---|
| t524 | 4 17 |
| t528 | 4 - |

### 2.3.5.3 *agr* typing animal *S. aureus*

The *agr* group I was the most frequent *agr* group among the selected *S. aureus* of animal origin followed by group II (41%) and group III (4%). Only a single strain was *agr* group IV (Figure 2.22).

Figure 2.22 *agr* groups of animal *S. aureus*



### 2.3.6 Concordance between molecular typing methods.

Using MLST data as a reference method, an evaluation of the concordance between typing methods for the animal *S. aureus* strain collection was determined. Regarding the isolates of singleton clonal complexes CC22, CC25 and CC47 typing results were identical for *spa* typing, PFGE, RS-PCR and *agr* typing (Table 2.29). The CC97 contained ST71, ST97 and ST815. These STs belonged to six different *spa* types of which four were clustered within *spa* CC267. *spa* types t524 and t528, both ST71, contained less than five *spa* repeats and were excluded from BURP analysis. Four PF groups (PF D, PF G, PF P and PF U) and five PR types (PR3, PR6, PR7, PR8 and PR23) were represented in this CC. Isolates belonging to PF group D subtype 9 and PR23 accounted for 45% of all isolates within CC97. All isolates within CC97 were *agr* group I. The singleton clonal complex CC5 was represented by *spa* CC2, containing *spa* types t002 and t306, two PF groups, PF A and PF C, four RS-PCR types, PR 1, PR2, PR14 and PR20, and all belonged to *agr* group II. Three of the seven isolates within CC5 belonged to *spa* CC2, PF group A and PR type 1. Nine isolates comprised the singleton clonal complex CC9. This CC was represented by *spa* CC1430, containing *spa* types t337, t1419 and t1430, a single PF group, group T, that formed a single dendogram cluster of isolates at a >80% similarity value, two PR types, PR4 and PR127iv/vi/vii and all belonged to *agr* group II. The PF subtype T9 however, although clustering with the other PF T subtypes at a similarity level of 74%, belonged to ST15, *spa* type t84, PR type 127i and *agr* group II. A single isolate belonging to ST188 clustered within PF group D, *spa* CC267 and belonged to PR type 3 and *agr* group I. These data are consistent with the inclusion of this strain within the MLST CC97. This ST is a triple locus variant of both ST97 and ST71 and a four locus variant of ST815 and was therefore not clustered within CC97 (Table 2.29). Three isolates formed CC692. This CC was represented by *spa* types t1422 and a new *spa* type, a single PF group, group M, that

formed a single dendogram cluster at a >70% similarity level and belonged to PR type 3 and *agr* group I. Three isolates clustered at an 80% similarity value in PF group J. These three isolates, belonged to ST385 and ST816, which differed at all seven loci, *spa* types t213 and t1429 which had only a single *spa* repeat in common, and three distinct PR types (Table 2.29).

Table 2.29 MLST, *spa* and *agr*, PF and PR types of animal isolates

| Animal source | CC | ST | arcC | aroE | glpF | gmk | pta | tpi | ygiL | *spa* type | *spa* repeat pattern alignment | | PF group | PF subtype | PR type |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | Kreiswerth nomenclature | Ridom nomenclature | | | |
| Chicken | 5 | 5 | 1 | 4 | 1 | 4 | 12 | 1 | 10 | t306 | TJMBMDMGMMK | 26-23-17-34-17-20-17-12-17-17-16 | A | A5 | 2 |
| Chicken | | 5 | 1 | 4 | 1 | 4 | 12 | 1 | 10 | t002 | TJMBMDMGMK | 26-23-17-34-17-20-17-12-17 - 16 | A | A7 | 1 |
| Chicken | | 5 | 1 | 4 | 1 | 4 | 12 | 1 | 10 | t002 | TJMBMDMGMK | 26-23-17-34-17-20-17-12-17 - 16 | A | A7 | 20 |
| Chicken | | 5 | 1 | 4 | 1 | 4 | 12 | 1 | 10 | t002 | TJMBMDMGMK | 26-23-17-34-17-20-17-12-17 - 16 | A | A7 | 1 |
| Phillips Goose Hawk | | 5 | 1 | 4 | 1 | 4 | 12 | 1 | 10 | t002 | TJMBMDMGMK | 26-23-17-34-17-20-17-12-17 - 16 | A | A2 | 1 |
| Saker hawk | | 5 | 1 | 4 | 1 | 4 | 12 | 1 | 10 | t306 | TJMBMDMGMMK | 26-33-17-34-17-20-17-12-17-17-16 | A | A10 | 14 |
| Cow | | 5 | 1 | 4 | 1 | 4 | 12 | 1 | 10 | t002 | TJMBMDMGMK | 26-23-17-34-17-20-17-12-17 - 16 | C | C1 | 1 |
| Pig | 9 | 9 | 3 | 3 | 1 | 1 | 1 | 1 | 10 | t337 | UKJJAGJAB | 07-16-23-23-02-12-23-02-34 | T | T8 | 4 |
| Pig | | 9 | 3 | 3 | 1 | 1 | 1 | 1 | 10 | t1428 | UKJAGJAB | 07-16-23 - 02-12-23-02-34 | T | T8 | 4 |
| Milk | | 9 | 3 | 3 | 1 | 1 | 1 | 1 | 10 | t337 | UKJJAGJAB | 07-16-23-23-02-12-23-02-34 | T | T1 | 127iv/vi/vii |
| Milk | | 9 | 3 | 3 | 1 | 1 | 1 | 1 | 10 | t1419 | UJAGJAB | 07 - 23 - 02-12-23-02-34 | T | T6 | 4 |
| Milk | | 9 | 3 | 3 | 1 | 1 | 1 | 1 | 10 | t337 | UKJJAGJAB | 07-16-23-23-02-12-23-02-34 | T | T7 | 127iv/vi/vii |
| Cow | | 9 | 3 | 3 | 1 | 1 | 1 | 1 | 10 | t337 | UKJJAGJAB | 07-16-23-23-02-12-23-02-34 | T | T3 | 4 |
| Cow | | 9 | 3 | 3 | 1 | 1 | 1 | 1 | 10 | t337 | UKJJAGJAB | 07-16-23-23-02-12-23-02-34 | T | T7 | 4 |
| Cow | | 9 | 3 | 3 | 1 | 1 | 1 | 1 | 10 | t337 | UKJJAGJAB | 07-16-23-23-02-12-23-02-34 | T | T4 | 4 |
| Cow | | 9 | 3 | 3 | 1 | 1 | 1 | 1 | 10 | t337 | UKJJAGJAB | 07-16-23-23-02-12-23-02-34 | T | T2 | 4 |
| Cow | | 15 | 13 | 13 | 1 | 1 | 12 | 11 | 13 | t84 | UJGBBGGJAGJ | 07-23-12-34-34-12-12-23-2-12-23 | T | T9 | 127i |
| Pig | 22 | 22 | 7 | 6 | 1 | 5 | 8 | 8 | 6 | t223 | TJEJCMOMOKR | 26-23-13-23-05-17-25-17-25-16-28 | L | L1 | E15 |
| Milk | | 22 | 7 | 6 | 1 | 5 | 8 | 8 | 6 | t223 | TJEJCMOMOKR | 26-23-13-23-05-17-25-17-25-16-28 | L | L2 | E15 |
| Horse | 25 | 25 | 4 | 1 | 4 | 1 | 5 | 5 | 4 | t078 | ZFGU$_2$DMGGM | 04-21-12-41 - - 20-17-12-12-17 | I | I1 | 138 |
| Parrot | | 25 | 4 | 1 | 4 | 1 | 5 | 5 | 4 | t1423 | ZFGU$_2$GU$_2$DMGGM | 04-21-12-41-12-41-20-17-12-12-17 | I | I1 | 138 |
| Horse | | 30 | 2 | 2 | 2 | 2 | 6 | 3 | 2 | t012 | WGKAKAOMQQ | 15-12-16-02-16-02-25-17-24-24 | S | S1 | E16 |
| Unknown | 45 | 47 | 10 | 11 | 8 | 6 | 10 | 3 | 2 | t026 | XKB | 08-16-34 | N | N1 | 111 |
| Cow | | 47 | 10 | 11 | 8 | 6 | 10 | 3 | 2 | t026 | XKB | 08-16-34 | N | N1 | 111 |

Table 2.29 MLST, *spa* and *agr*, PF and PR types of animal isolates

| Animal source | CC | ST | arcC | aroE | glpF | gmk | pta | tpi | ygiL | spa type | *spa* repeat pattern alignment | | PF group | PF subtype | PR type |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | Kreiswerth nomenclature | Ridom nomenclature | | | |
| Milk | | 71 | 18 | 1 | 1 | 1 | 1 | 5 | 3 | t528 | Z | 04 - | D | D8 | 8 |
| Milk | | 71 | 18 | 1 | 1 | 1 | 1 | 5 | 3 | t524 | ZM | 04 17 | D | D6 | 3 |
| Milk | | 71 | 18 | 1 | 1 | 1 | 1 | 5 | 3 | t524 | ZM | 04 17 | U | U1 | 7 |
| Milk | | 71 | 18 | 1 | 1 | 1 | 1 | 5 | 3 | t524 | ZM | 04 17 | P | P2 | 7 |
| Milk | | 71 | 18 | 1 | 1 | 1 | 1 | 5 | 3 | t524 | ZM | 04 17 | D | D17 | 7 |
| Milk | | 97 | 3 | 1 | 1 | 1 | 1 | 5 | 3 | t224 | UJGFMBPB | 07-23-12-21-17-34 - - - - 33-34 | G | G1 | 6 |
| Milk | | 97 | 3 | 1 | 1 | 1 | 1 | 5 | 3 | t224 | UJGFMBPB | 07-23-12-21-17-34 - - - - 33-34 | D | D16 | 7 |
| Milk | 97 | 97 | 3 | 1 | 1 | 1 | 1 | 5 | 3 | t224 | UJGFMBPB | 07-23-12-21-17-34 - - - - 33-34 | G | G1 | 6 |
| Milk | | 97 | 3 | 1 | 1 | 1 | 1 | 5 | 3 | t527 | UJGFMBBBBBPB | 07-23-12-21-17-34-34-34-34-34-33-34 | D | D5 | 3 |
| Milk | | 97 | 3 | 1 | 1 | 1 | 1 | 5 | 3 | t224 | UJGFMBPB | 07-23-12-21-17-34 - - - - 33-34 | D | D21 | 7 |
| Milk | | 97 | 3 | 1 | 1 | 1 | 1 | 5 | 3 | t267 | UJGFMBBBPB | 07-23-12-21-17-34-34-34 - - 33-34 | D | D6 | 7 |
| Milk | | 97 | 3 | 1 | 1 | 1 | 1 | 5 | 3 | t224 | UJGFMBPB | 07-23-12-21-17-34 - - - - 33-34 | D | D14 | 3 |
| Cow | | 97 | 3 | 1 | 1 | 1 | 1 | 5 | 3 | t1421 | UJG(new spa sequence)BBPB | 07-23-12-118 - - - - 34-34-33-34 | D | D9 | 23 |
| Milk | | 188 | 3 | 1 | 1 | 8 | 1 | 1 | 1 | t1418 | UJGFMEBBPB | 07-23-12-21-17-13 - - 34-34-33-34 | D | D2 | 3 |
| Milk | | 815 | 3 | 1 | 109 | 1 | 1 | 5 | 3 | t527 | UJGFMBBBBBPB | 07-23-12-21-17-34-34-34-34-34-33-34 | D | D5 | 3 |
| Pig | | 133 | 6 | 66 | 46 | 2 | 7 | 50 | 18 | t1428 | D2GFMJEMMJEMMMMJQ | 03-12-21-17-23-13-17-17-23-13-17-17-17-17-23-24 | Q | Q1 | 12 |
| Lamb | | 133 | 6 | 66 | 46 | 2 | 7 | 50 | 18 | t1428 | D2GFMJEMMJEMMMMJQ | 03-12-21-17-23-13-17-17-23-13-17-17-17-17-23-24 | Q | Q2 | 12 |
| Pheasant | | 385 | 1 | 89 | 1 | 1 | 4 | 5 | 11 | t213 | UJGFQPLM | 07-23-12-21-24-33-22-17 | J | J1 | 11 |
| Chicken | | 692 | 12 | 89 | 1 | 1 | 4 | 5 | 90 | new | TJGBFMBOM | 26-23-12-34-21-17-34-25-17 | M | M3 | 3 |
| Cow | | 773 | 16 | 16 | 12 | 1 | 13 | 13 | 15 | t1420 | ZDMJ(new spa sequence)DMO | 04-20-17-23-117-20-17-25 | O | O2 | 19 |
| Chicken | | 774 | 4 | 1 | 1 | 39 | 1 | 1 | 1 | t177 | TJFKBPE | 26-23-21-16-34-33-13 | D | D28 | 5 |
| Pheasant | | 814 | 12 | 89 | 108 | 1 | 4 | 5 | 90 | t1422 | TJGBFMBBOM | 26-23-12-34-21-17-34-34-25-17 | M | M5 | 3 |
| Chicken | | 814 | 12 | 89 | 108 | 1 | 4 | 5 | 90 | t1422 | TJGBFMBBOM | 26-23-12-34-21-17-34-34-25-17 | M | M5 | 3 |
| Horse | | 816 | 18 | 140 | 45 | 2 | 7 | 14 | 7 | t1429 | WMBBBPB | 15-17-34-34-34-33-34 | J | J2 | 17 |
| Cow | | 816 | 18 | 140 | 45 | 2 | 7 | 14 | 7 | t1429 | WMBBBPB | 15-17-34-34-34-33-34 | J | J2 | 1 |

### 2.3.7 MLST, *spa* and *agr* typing of selected human *S. aureus*

Twenty eight human *S. aureus* were selected for characterization by MLST, *spa* typing, and *agr* typing, and correlation with animal *S. aureus* strains. These isolates were chosen based on their similarity to isolates from animals based on RS-PCR and PFGE patterns.

A total of 15 STs were detected among the 28 human *S. aureus* (Table 2.30). eBurst (eBurst Ver3) analysis two minor groups (CC5 and CC8), containing five isolates each, and 11 singleton STs (Table 2.31). Seven of the STs detected (ST5, ST8, ST22, ST30, ST45, ST125 and ST247) have been previously described with all five of the major MRSA lineages (CC5, CC8, CC22, CC30 and CC45).

Table 2.30 PF, PR, MLST, *spa* and *agr* types of human isolates

| Specimen No. | RS-PCR type | PF TYPE | MLST | | | | | | | | *spa* repeat sequence | *spa* type | *agr* group |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | ST | arcC | aroE | glpF | gmk | pta | tpi | ygiL | | | |
| 04.1039.F | 116/124 | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 07-23-21-16-34-33-13 | t127 | III |
| 04.1000.N | 132/154 | | 5 | 1 | 4 | 1 | 4 | 12 | 1 | 10 | 26-23-17-34-17-20-17-12-17-16 | t002 | II |
| 03.5940.H | 132/105 | | 5 | 1 | 4 | 1 | 4 | 12 | 1 | 10 | 26-23-17-34-17-20-17-12-17-16 | t002 | II |
| 00.8498.T | | 105-33 | 5 | 1 | 4 | 1 | 4 | 12 | 1 | 10 | 26-23-17-17-20-17-12-17-16 | t579 | II |
| 05.1957.Z | | 105 | 5 | 1 | 4 | 1 | 4 | 12 | 1 | 10 | 26-23-17-34-20-17-12-17-16 | t311 | II |
| 01.7848.C | 108v | | 8 | 3 | 3 | 1 | 1 | 4 | 4 | 3 | 11-19-12-21-17-34-24-34-22-25 | t008 | I |
| 01.9898.D | 150 | | 8 | 3 | 3 | 1 | 1 | 4 | 4 | 3 | 11-17-34-24-34-22-25 | t190 | I |
| 03.5593.Y | 105iii/108v | | 8 | 3 | 3 | 1 | 1 | 4 | 4 | 3 | 11-19-12-05-17-34-24-34-22-25 | t064 | I |
| 05.1117.W | | 124 | 8 | 3 | 3 | 1 | 1 | 4 | 4 | 3 | 11-19-12-21-17-34-24-34-22-25 | t008 | I |
| 01.7817.X | 127 | | 15 | 13 | 13 | 1 | 1 | 12 | 11 | 13 | 07-23-12-34-34-12-12-23-02-12-23 | t84 | II |
| 01.10028.D | 127i | | 15 | 13 | 13 | 1 | 1 | 12 | 11 | 13 | 07-23-12-34-12-12-23-02-12-23 | t346 | II |
| 01.1420.K | | 127 | 15 | 13 | 13 | 1 | 1 | 12 | 11 | 13 | 07-23-12-34-34-12-23-02-12-23 | t085 | II |
| 03.7849.X | 127iii/v | | 20 | 4 | 9 | 1 | 8 | 1 | 10 | 8 | 07-06-17-21-34-34-22-34 | t164 | I |
| 05.1111.R | 15 | 15 | 22 | 7 | 6 | 1 | 5 | 8 | 8 | 6 | 26-23-13-31-05-17-25-17-25-16-28 | t1433 | I |
| 05.1060.P | 15 | 15 | 22 | 7 | 6 | 1 | 5 | 8 | 8 | 6 | 26-23-13-31-05-17-25-17-25-16-28 | t1433 | I |
| 04.1381.H | 109/112 | | 30 | 2 | 2 | 2 | 2 | 6 | 3 | 2 | 15-12-16-02-16-02-25-17-24-24 | t012 | III |
| 05.1058.T | 16 | 16 | 30 | 2 | 2 | 2 | 2 | 6 | 3 | 2 | 15-12-17-16-02-16-02-16-02-25-17-24 | t1432 | III |
| 05.1022.F | 16 | 16 | 30 | 2 | 2 | 2 | 2 | 6 | 3 | 2 | 15-12-17-16-02-16-02-16-02-25-17-24 | t1432 | III |
| 02.4615.V | 111 | | 45 | 10 | 14 | 8 | 6 | 10 | 3 | 2 | 09-02-16-34-13-17-34-16-34 | t065 | I |
| 05.1017.P | | 111 | 45 | 10 | 14 | 8 | 6 | 10 | 3 | 2 | 08-16-02-16-34-34-17-34-16-34 | t050 | I |
| 03.9238.Y | 137 | | 49 | 14 | 16 | 11 | 2 | 13 | 12 | 14 | 04-20-17-20-17-17-31-31-24-17-17-25 | t1431 | II |
| 02.6322.D | 133 | | 109 | 3 | 27 | 1 | 1 | 1 | 1 | 10 | 07-16-12-23-34 | t209 | II |
| 01.1652.H | | 125 | 123 | 6 | 5 | 6 | 2 | 7 | 17 | 19 | 14-4413-12-17-17-17-17-23-18 | t171 | IV |
| 01.7081.W | | 105 | 125 | 1 | 4 | 1 | 4 | 12 | 1 | 54 | 26-23-17-34-17-20-17-12-17 | t067 | I |
| 00.6064.G | | 118 | 247 | 3 | 3 | 1 | 12 | 4 | 4 | 16 | 04-21-12-41-20-17-12-12-17 | t051 | I |
| 01.8651.D | 138 | | 25 | 4 | 1 | 4 | 1 | 5 | 5 | 4 | 04-21-12-41-20-17-12-12-17 | t078 | I |
| 02.7658.H | | 138 | 25 | 4 | 1 | 4 | 1 | 5 | 5 | 4 | 11-19-21-12-21-17-34-24-34-22-25 | t78 | I |
| 04.4058.K | 127iii/vi/vii | | 789 | 3 | 4 | 1 | 4 | 4 | 6 | 3 | 07-23-21-17-34-12-23-02-12-23 | t091 | I |

Table 2.31 Minor groups and singleton STs detected among the 28 human *S. aureus*

| Singletons |
| --- |
| ST1, ST15, ST20, ST22, ST25, ST30<br><br>ST45, ST49, ST109, ST123, ST789 |
| **Minor groups** |
| ST5----------ST125<br><br>ST8----------ST247 |

---------- indicates a SLV difference

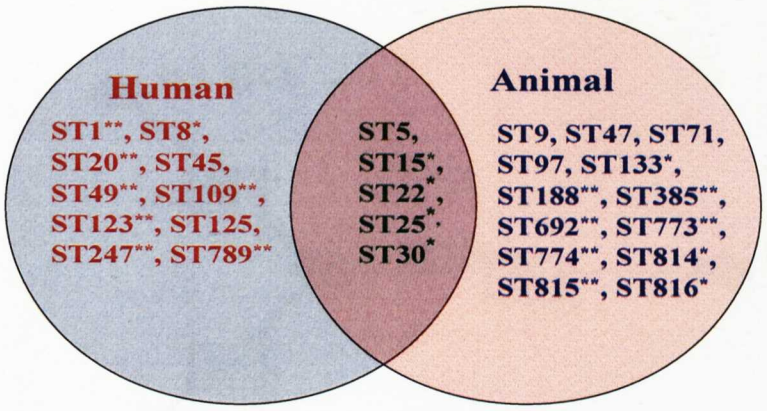
Sequence analysis of the X region of the *spa* gene resulted in the detection of 23 *spa* types varying in length between five (t209) and 12 (t1431 and t1432) repeats including three types not found in the Ridom database (Table 2.25). Five *spa* types, t002, t008, t078, t1432 and t1433, were detected more than once. The new *spa* type t1433 has subsequently been reported in an isolate from the Netherlands (Ridom SpaServer database last viewed 16.12.07). Fifty percent of human isolates belonged to *agr* group I, and was the most frequently detected *agr* group, followed by group II (32.1%), and group III (14.3%). Only a single strain (ST123) belonged to *agr* group IV (Table 2.25).

These data were combined with the data generated from the animal *S. aureus* isolates and compared to determine strain similarity between isolate populations.

### 2.3.8 Correlation of characterization data between animal and human *S. aureus* isolates

Of the 28 STs identified, among the 77 isolates examined, 13 STs were found only among animal strains, 10 STs only among human strains and five STs (ST5, ST15, ST22, ST25 and ST30) common to both populations (Figure 2.23). A sixth ST, ST45, detected in a human isolate was a SLV of ST47 found in an animal isolate. eBURST analysis of the combined animal and human ST datasets one major CC (CC97), three minor CCs (CC5, CC47, and CC814) and 18 singleton STs. Fifty percent of the STs identified were single isolate and nine STs comprised of between two and nine isolates. Two of the minor CCs (CC5 and CC47) contained isolates from both human and animal sources. CC5 comprised of 12 isolates, seven animal and five human, and included ST5 and the SLV ST125. CC47 was comprised of four isolates, two human and two animal, and included ST45, only identified from human isolates, and the SLV ST47, only identified from animal sources.

Figure 2.23 STs identified among human and animal *S. aureus* including those common to both strain collections.

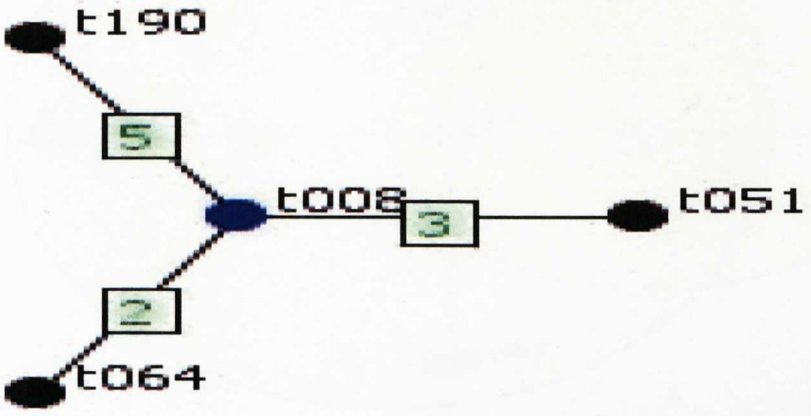



* Singleton STs, ** Singleton STs that were also single isolate

### 2.3.8.1 *spa* typing and based upon repeat pattern analysis (BURP)

Using the algorithm BURP, Ridom StaphType™ software, *spa* types were grouped together if the calculated cost between members of a group was less than or equal to eight and formed eight clusters (*spa* CC2, *spa* CC8, *spa* CC12, *spa* CC78, *spa* CC84, *spa* CC223, *spa* CC267 and *spa* CC1430) and 15 singleton *spa* types[414]. The most frequent *spa* types were t002 (six animal and two human isolates), t337 (seven animal isolates), and t224 (five animal isolates). Since clustering parameters excluded *spa* types shorter than five repeats, three types (t026, t524 and t528) were excluded from BURP grouping. Five clusters, *spa* CC2, *spa* CC12, *spa* CC78, *spa* CC84 and *spa* CC223 contained isolates from human and animal sources. *spa* CCs 267 and 1430 (Figures 2.18 and 2.19) were exclusive to animal isolates and *spa* CC8 contained only human isolates (Figure 2.24). *spa* CC8 was represented by five human *S. aureus* isolates. Four isolates, t008 (two isolates), t064 (single isolate) and t190 (single isolate) were ST8. The remaining isolate belonged to *spa* type t051 and was ST247, a DLV of ST8. All isolates clustered at a calculated cost of ≤5 (Figure 2.24).

Figure 2.24 BURP analysis of the identified *spa* types in *spa* CC8

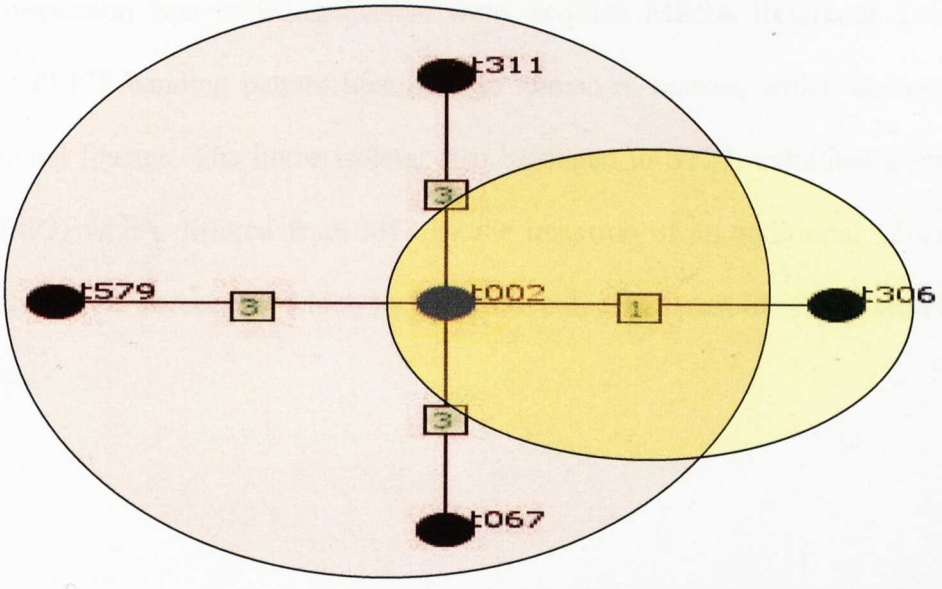


*spa* CC2 contained five *spa* types, with only t002 common to human and animal isolates (Table 2.32). These isolates grouped at a calculated cost of ≤3 (Figure 2.25).

Table 2.32 Correlation between *spa* type and ST in *spa* CC2

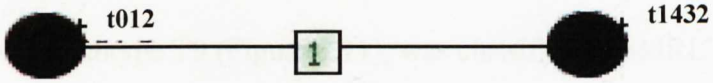
| *spa* type | ST | Number of isolates | |
|---|---|---|---|
| | | Animal | Human |
| t002 | 5 | **2** | **5** |
| t067 | 125 | | 1 |
| t306 | 5 | 2 | |
| t311 | 5 | | 1 |
| t579 | 5 | | 1 |

Figure 2.25 BURP analysis of the identified *spa* types in *spa* CC2



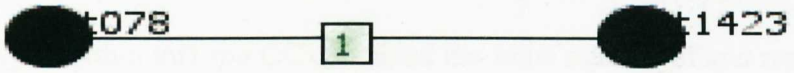
*spa* CC12 was represented by three isolates, two human isolates (t1432) and a single companion animal isolate (t012), clustering at a calculated cost of ≤1 (Figure 2.26). These isolates had identical ST (ST30) and were grouped together by RS-PCR (PR16) and PFGE (PF 16).

Figure 2.26 BURP analysis of the identified *spa* types in *spa* CC12




*spa* CC78 was represented by four isolates; two companion animal isolates, one horse from Southwest Scotland and one parrot from Highland, and two human isolates. Three isolates belonged to t078, this included both human isolates (PF 138 and ST25) and the parrot isolate (Figure 2.27). Both animal isolates belonged to PF subtype I1, which on visual inspection was indistinguishable from Scottish MRSA Reference Laboratory (SMRL) PF138 banding pattern identified in human *S. aureus*, which belongs to the ST25 clonal lineage. The horse isolate, also belonged to ST25, exhibited a novel *spa* type (t1423) which differed from t078 by the insertion of an additional 12-41 repeat within the repeat succession which resulted in a calculated cost of ≤1 between the two *spa* types.

Figure 2.27 BURP analysis of the identified *spa* types in *spa* CC78




*spa* CC84 was represented by four isolates, three human and a single bovine isolate from Grampian region, and by three *spa* types, including t084 the predicted founder of this *spa* CC (Figure 2.28). The human isolates belonged to PR/PF type 127 using the SMRL nomenclature and belonged to the same ST as the animal isolate (Table 2.33). On visual inspection, of the RS-PCR and PFGE banding patterns, the animal isolate belonging to PF subtype T9 (Figure 2.11), was classified as SMRL PR 127 and PF127.

Figure 2.28 BURP analysis of the identified *spa* types in *spa* CC84

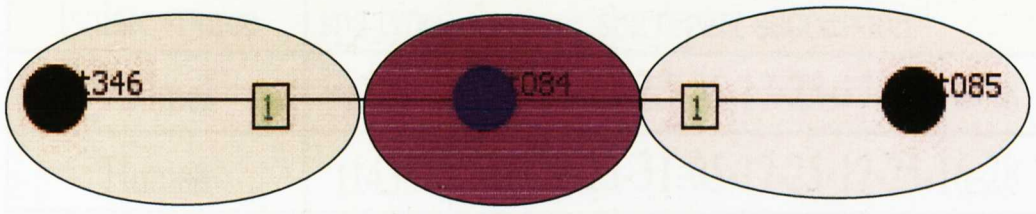



Table 2.33 Burp analysis of the identified *spa* types in *spa* CC84

| ST | *spa* type | No isolates | |
|---|---|---|---|
| | | Animal | Human |
| 15 | t084 | 1 | 1 |
| | t085 | | 1 |
| | t346 | | 1 |

Isolates belonging to *spa* CC223 included two human and two animal isolates, one pig from Highland and one milk from Grampian region (Figure 2.29). Although both *spa* types within this *spa* CC contained the same number of *spa* repeats, repeat number four in the repeat succession differed with the deletion of r25 and insertion of r31 within t1433 (Table 2.34).

Figure 2.29 BURP analysis of the identified *spa* types in *spa* CC223


Table 2.34 *spa* repeat variation in isolates belonging to *spa* CC223

| Isolate source | spa type | spa repeat succession |
|---|---|---|
| Animal | t223 | 26-23-13-**23**-05-17-25-17-25-16-28 |
| Human | t1433 | 26-23-13-**31**-05-17-25-17-25-16-28 |

The animal strains, belonging to PF group L, on visual inspection were classified using the SMRL nomenclature as PR type 15 and PF type 15 and were indistinguishable from human isolates.

Using the *spa* typing parameters described in the materials and methods, all isolates were grouped together as expected regarding their evolutionary origin, as reflected by MLST analysis, although in most cases a variety of *spa* types corresponded to a single MLST ST (Table 2.35).

Table 2.35 Distribution of *spa* types among animal and human *S. aureus* isolates where isolates belonging to the same ST had different *spa* types

| ST | *spa* types |
|---|---|
| 5 | t002, t067, t306, t311, t579, |
| 8 | t008, t064, t190 |
| 9 | t337, t1419, t1430 |
| 15 | t084, t085, t346 |
| 22 | t223, t1433 |
| 71 | t524, t528 |
| 97 | t224, t267, t527, t1421 |

### 2.3.9 Relationships between ST, *spa* type, *agr* type and PR/PF types among animal and human *S. aureus* isolates

Five STs (ST5, ST15, ST22, ST25 and ST30) were common to both human and animal *S. aureus* isolates examined in this study. Although ST45 (human isolate) and ST47 (animal isolate) were not common to both isolate collections these strains both belong to MLST CC45. These STs together with ST5, ST22, and ST30 represent four of the five major contemporary epidemic *S. aureus* clonal lineages. A high level of concordance between the four typing and clustering methods was demonstrated (Table 2.36). As the BURP algorithm excludes *spa* types with less than 5 repeats, isolates belonging to ST45 and ST47 (PF 111 and PF subtype N1 respectively and both *agr* type I) were not clustered together by *spa* type. The *spa* type t026, belonging to ST47, shares *spa* repeats r8, r16 and r34 with *spa* types t050 and t060 and could be clustered based on visual inspection of the repeat successions (Table 2.37).

Table 2.36 Correlation between ST, *spa* type, *agr* type and PR/PF types common to animal and human *S. aureus*

| ST | *spa* CC | *spa* types | *agr* type | PR/PF type | |
|---|---|---|---|---|---|
| | | | | Human | Animal |
| 5/125 | CC2 | t002, t306 , t311, t579 | II | 105 | 105 |
| 15 | CC84 | t084, t085, t346 | II | 127 | 127 |
| 22 | CC223 | t223, t1433 | I | 15 | 15 |
| 25 | CC78 | t078, t1423 | I | 138 | 138 |
| 30 | CC12 | t012, t1432 | III | 16 | 16 |
| 45/47 | * | t026, t050, t060 | I | 111 | 111 |

* BURP analysis excludes *spa* types with <5 repeats therefore did could not place these *spa* types within a *spa* CC.

Table 2.37 Comparison of ST and *spa* types of PF type 111/N1 isolates

| Isolate source | PF type | ST | Allelic profile | *spa* type | |
|---|---|---|---|---|---|
| | | | | t | Repeat succession |
| Human | 111 | 45 | 10-14-8-6-10-3-2 | t065 | 09-02-16-34-13-17-34-**16-34** |
| | | | | t050 | **08**-16-02-16-34-34-17-34-**16-34** |
| Animal | N1 | 47 | 10-**11**-8-6-10-3-2 | t026 | **08-16-34** |

There was good overall correlation between the five molecular characterization methods used (RS-PCR, PFGE, *spa* typing, MLST and *agr* typing). Based on the visual inspection of the RS-PCR and PFGE banding patterns, of the human and animal isolate collections, three PR/PF types common to both groups belonged to different STs, *spa* types and for two out of three *agr* groupings (Table 2.38).

Table 2.38 Incongruence between PR/PF types, ST, *spa* type and *agr* group of human and animal isolates

| PR/PF type | Source | ST | Allelic profile | *spa* type | | *agr* group |
|---|---|---|---|---|---|---|
| | | | | t | Repeat succession | |
| 124 | Human | 8 | 3-3-1-1-4-4-3 | t008 | 11-19-12-21-17-34-24-34-22-25 | I |
| | Animal | 774 | 4-1-1-39-1-1-1 | t177 | 26-23-21-16-34-33-13 | III |
| 125 | Human | 123 | 6-5-6-2-7-17-19 | t171 | 14-44-13-12-17-17-17-17-23-18 | IV |
| | Animal | 773 | 16-16-12-1-13-13-15 | t1420 | 04-20-17-23-117-20-17-25 | IV |
| 137 | Human | 49 | 14-16-11-2-13-12-14 | t1431 | 04-20-17-20-17-17-31-31-24-17-17-25 | II |
| | Animal | 133 | 6-66-46-2-7-50-18 | t1428 | 03-12-21-17-23-13-17-17-23-13-17-17-17-17-23-24 | I |

## 2.4 Discussion

The epidemiology of infectious disease relies on typing methods as tools for the characterization and discrimination of isolates based on either their genotypic or phenotypic characteristics[129]. These methods can be used to establish clonal relationships between strains and to trace the geographical dissemination of bacterial clones. While there are numerous methods for typing *S. aureus*, not all methods divide groups of strains in a similar fashion. The methods used in this study have been used widely for the characterization of *S. aureus* from humans but have been infrequently used to characterize *S. aureus* from a range of livestock, companion or wild animals[270,301,401].

### 2.4.1 Characterization methods

#### 2.4.1.1 Phenotypic characterization

Phenotypic characterization of *S. aureus* isolates by the antibiotic resistance pattern has been used as a first step in strain monitoring. It is a simple, fast and inexpensive method of survey but lacks sufficient discriminatory power. This lack of discriminatory power was apparent in this study where the antibiogram method identified 32 resistance phenotypes with a discriminatory value of 88.2%. Although, this level of discrimination was almost three times higher than that reported by Aarestrup *et al* in an investigation of *S. aureus* from bovine mastitis in Denmark[7]. The poor discriminatory power of antibiogram typing is attributed to the fact that two strains with no epidemiological link can have the same antibiogram while two isolates of the same clone can acquire and lose plasmid mediated antibiotic resistance and exhibit different phenotypes[94].

Therefore, like the use of antibiograms to characterize human *S. aureus*, antibiograms are an unsuitable method for the characterization of *S. aureus* isolated from animals.

Phage typing has been used for typing human *S. aureus* and has been the standard method for typing *S. aureus* from cases of bovine mastitis since the early 1960's [42,381,497]. In studies by Slanetz *et al* [399], St George *et al* [412] and Frost *et al* [145] the level of non typable isolates from bovine sources, when using the 23 phages of the international phage set (IPS) for typing human strains of *S. aureus*, was high. The frequency of non typable *S. aureus* isolates from animals in this study was also high at 24% resulting in the lowest discriminatory value (83.5%) of all the methods used to characterize *S. aureus* isolates from animals. Studies by Vintov *et al*, of bovine *S. aureus* from 10 different countries, has shown a similarly high (23%) level of phage nontypability[458]. In contrast, Aarestrup *et al*[7], Zadoks *et al*[497] and Capita *et al*[60] have all reported very high levels of phage typeability (93%) for *S. aureus* isolates from bovine and poultry and concluded that the use of the internationally recognized and well investigated human set of phages gives the possibility to compare the occurrence of different phage types among livestock animals.

There was an overall low level of correlation with other typing techniques indicating a poor level of concordance between phage typing and DNA-based techniques a point also noted by Grundmann *et al*[165]. Due to the many drawbacks, which include limited typeability, limited technical reproducibility and a lack of biological reproducibility, phage typing is also not a suitable method for the characterization of *S. aureus* from animals and has been superseded by DNA-based typing techniques.

### 2.4.1.2 Molecular characterization

Amplification of the variable region between the 16S and 23S rRNAs by RS-PCR[83] has been shown to successfully characterize *S. aureus* isolates from human clinical sources[205]. This method is based on the observation of PCR products corresponding to the short intergenic regions between the genes for the ribosomal 16S and 23S RNA[159]. Dolzani *et al* reported that RS-PCR had a 100% typeability level, was reproducible and almost as discriminatory as PFGE for outbreak isolates of MSSA and MRSA with a discriminatory value of 92%[111]. In this study RS-PCR was able to type all isolates, resulting in 27 different PR types, and a discriminatory value (86.2%) lower than that of antibiogram resistance phenotype and PFGE. In an attempt to increase the discriminatory capacity of this method some authors have used endonuclease digestion of the amplified RS-PCR products[205]. Couto *et al* suggest that the restriction of amplicons with restriction enzymes is not necessary[83]. Kumari *et al* also came to the same conclusion indicating that the endonuclease digested fragments are very small and present difficulties in the interpretation of banding patterns[243]. The low discriminatory capacity of this technique renders it ill suited for the characterization of *S. aureus* isolated from animals.

PFGE, of SmaI-digested genomic DNA, is the most commonly used method for studying the microepidemiology (local or short term) and macroepidemiology (national, continental or long term) of *S. aureus* from humans[172]. It has also proven invaluable for the characterization of *S. aureus* isolates from humans, cases of bovine[497,498] and ovine[456] mastitis, from infection in poultry[286,360,361] and pigs[193], and MRSA from companion animals[415,467]. The criteria for interpreting PFGE band patterns, as published by Tenover *et al*, categorizes isolates as indistinguishable (no band

differences), closely related (one to three band differences), possibly related (four to six band differences), or different (more than six band differences) and was developed to analyze discrete sets of isolates obtained during epidemiologic studies of potential outbreaks spanning short periods of time (1 to 3 months) and a narrow geographic distribution[424]. It has also been argued that the stability of PFGE may be sufficient for its reliable application to long term epidemiological studies[43,172]. Although, some authors have argued that the stabilities of PFGE markers may be insufficiently reliable for long term studies: high degrees of genetic variation leading to multiple PFGE banding patterns have been observed for pandemic clones with long evolutionary histories. This has been attributed to heterologous selective pressure in the environment[13] and to the fact that large chromosomal fragments undergo faster variation than smaller ones that lead to a progressive drift of PFGE patterns over time[172].

All isolates could be typed using this technique and visual inspection of the banding patterns of the 233 animal *S. aureus* isolates resolved 74 pulsed field patterns resulting in a discriminatory value of 93.1%. This level of discrimination is consistent with data published by Cookson *et al*[80] and Hallin *et al*[172] for typing *S. aureus* from humans and Zadoks *et al*[497] and dos Santos *et al*[112] for typing *S. aureus* from bovine sources. The level of concordance between PFGE and RS-PCR was high. Although, combining both methods did not significantly increase the overall level of discrimination.

PFGE grouped all isolates into 20 groups, each containing between one and 19 subtypes, at a 70% cut-off value which equated to Tenover's criteria for possibly related (≤6 bands difference)[414]. Four PF groups (PF groups A, D, M and T) accounted for 84% of the isolates. There was a clear association between PF group and animal and geographic source. PF groups A and M were predominantly from avian sources from Northern Ireland and Highland region respectively, while PF groups D and T were from bovine sources the Grampian region and Southwest Scotland. In contrast, a recent work by Faria *et al* has shown that to meet the Tenover criteria for possibly related required a similarity cut-off of 80% and one of 95% for closely related (1-3 bands difference). The presence of these limited number of PF groups among this collection of isolates suggests that a limited number of clones are responsible for infection from avian and bovine sources. This is in agreement with the findings of others. Aires-de-Sousa *et al* and Cabral *et al* have both reported that only a limited number of clones were responsible for cases *S. aureus* mastitis in cattle, goats, sheep and buffalo, and infection in ostrich in Brazil[15,59]. Also Jørgensen *et al* and Mørk *et al* have observed that a limited number of *S. aureus* genotypes were responsible for bovine, ovine and caprine mastitis and that these genotypes were also isolated from bulk milk from these species[211,305].

Despite the practical disadvantages, which include the high set up cost, its labour intensiveness, the unique technical skills required and difficulties with inter-laboratory comparisons, PFGE was able to type all animal *S. aureus* and cluster them into epidemiologically related groups.

*spa* typing is a single polymorphic locus sequence based typing method[172]. The sequence variations in this region arise from deletion and duplication of the repetitive units and also by point mutation, duplications or deletions within the repeat succession[322]. Shopsin *et al* has noted that the mutational rate within this region was sufficiently low to allow for adequate discrimination between *S. aureus* strains[396]. Kahl *et al*[212] has confirmed the conclusion of Shopsin *et al* by investigating the mutational rate within the X region in 10 patients with cystic fibrosis who were persistently colonized with a single *S. aureus* clone, determined by PFGE. They calculated that one genetic event occurred every 70 months with deletions being most common.

The major advantage of *spa* typing over PFGE is its ability to be applied to both local and global epidemiological studies. While *spa* typing does not have the resolving power of PFGE subtyping it has several advantages in terms of speed, ease of use, ease of interpretation and data management and dissemination [239]. While all isolates could be typed by *spa*, de Sousa *et al* have reported that on rare occasions no amplification of the *spa* X region occurred. This lack of amplification is thought to occur due to sequence mutation within the primer binding region[12]. Koreen *et al*[239], typing *S. aureus* from humans, and Moodley *et al*[301], typing MRSA from companion animals, have both shown the same level of typeability as was seen in this study. Sequence analysis of the X region of the *spa* gene resulted in the detection of 25 distinct *spa* types including 10 novel types, among the 49 isolates tested, and had a discriminatory value of 95.4%. This high level of discrimination has also been shown in data published by Strommenger *et al*[414] on human and animal *S. aureus* and Faria *et al*[129] on *S. aureus* from humans form 19 different countries collected over a fifty year period. All *spa* types were classified into four *spa*CCs, 10 singleton *spa* types and three excluded by BURP analysis as they

had less than five *spa* repeats. *spa* typing was shown to be similarly discriminating and concordant with PFGE in the characterization of the animal isolates of *S. aureus* in this study.

MLST resolved eighteen different STs among the *S. aureus* isolates from animals including seven novel STs (ST385, ST692, ST773, ST774, ST814, ST815 and ST816) and three novel alleles (glpF 108, glpF 109, and aroE 140) and had a diversity value of 91.4%. Joo *et al* suggest that the identification of novel STs and alleles may be evidence of selection pressures that create specific localized variants of *S. aureus*[209]. Therefore, it would not be unusual for animal isolates to possess novel alleles compared to the sequences of those strains isolated from humans. They also suggest that there may be a limited cross-over of animal and human strains which could also explain the presence of novel STs and alleles, with the isolated populations diversifying independently. The identification of the novel STs ST692, a SLV of ST814, due to the presence of the novel allele glpF108, and ST385, a DLV of ST692, and ST815 a SLV of ST97 and a DLV of ST71 could indicate the presence of localized, independently diversifying, clonal variants within this strain collection. Alternatively Smith *et al* suggest that the presence of novel STs and alleles may be merely due to the investigation of new populations, and as most analyses have focused on human clinical isolates, increased sampling and further analysis of other *S. aureus* populations would be likely to yield novel alleles[401].

The initial findings of a study of human *S. aureus* strains by Feil *et al* and Day *et al*, based on MLST, showed *S. aureus* to be highly recombinogenic. Although this conclusion has now been amended due to sequencing errors[132]. The revised data set demonstrated that *S. aureus* has a predominantly clonal population structure, and the findings of this study on *S. aureus* isolated from animals agree with this finding.

MLST provides unambiguous results that are electronically portable and therefore suitable for global surveillance studies via the internet[81]. A major disadvantage of MLST, however, is that phylogenetic relationships and resolution of clones can be masked by the use of slowly evolving housekeeping genes. Zhang *et al* have suggested that more rapidly evolving genetic loci should be used to resolve deeper relationships and provide a higher level of strain discrimination[499].

DNA-based typing schemes came to very similar conclusions as PFGE and the combining of different techniques did not increase the discrimination of the *S.aureus* strains from animals to a large extent. This contrasts with data from Aarestrup *et al* and Smith *et al* who have shown an increase in the discriminatory power of phenotypic and genotypic methods when combined[7,401]. Moreover, it appears that PFGE patterns could theoretically predict MLST clonal complexes, pointing to the presence of a largely congruent phylogenetic signal in different regions of the staphylococcal genome[165].

The characterization of *S. aureus* from animals has shown high overall agreement between MLST, *spa* and PFGE typing methods. Previous work on bovine and human *S. aureus* isolates has also demonstrated high overall agreement between genotypic typing methods[401,497].

Four allelic groups of *agr* have been characterized in *S. aureus* (numbered I-IV). Wright *et al*, using five different genotyping methods to characterize *S. aureus* strains, concluded that their results were so strongly correlated with *agr* that the former could be used to predict the latter unequivocally[491]. Of the 49 isolates examined, by *agr* multiplex PCR, group I (53%) was the most prevalent, followed by *agr* II (40.8%), *agr* III (4.1%) and a single isolate *agr* IV. Gilot *et al* have shown an identical distribution of *agr* groups among *S. aureus* isolated from cattle[156]. In contrast to this clear prevalence of *agr* groups I and II in animal *S. aureus* strains, Jarraud *et al* have shown an even distribution of *agr* groups among *S. aureus* isolated from humans[201].

In a study by Wright *et al* they concluded that with exceedingly rare exceptions no MLST pattern occurred in more than one agr group. Data from this study has also shown that strains belonging to the same MLST type also belonged to the same agr group. They also suggest that the same holds true for PFGE and *spa* typing[491]. This was not the case here where isolates belonging to a common PF group or *spa* type could belong to different *agr* groups. The hypothesis of Wright *et al*, based on the observation that no multilocus sequence type generally occurs in more than one agr group, that the divergence of agr groups in *S. aureus* preceded the development of nucleotide polymorphisms currently used for strain typing and that the species was phylogenetically structured according to agr has now been refuted. Robinson and

colleagues have shown that Staphylococcal clonal complexes themselves can belong to at least two subspecies groups both containing *agr* I and III. Therefore, because of recombination, *agr* I and III are not monophyletic and the species cannot be phylogenetically structured according to *agr* (Figure 2.30)[358].

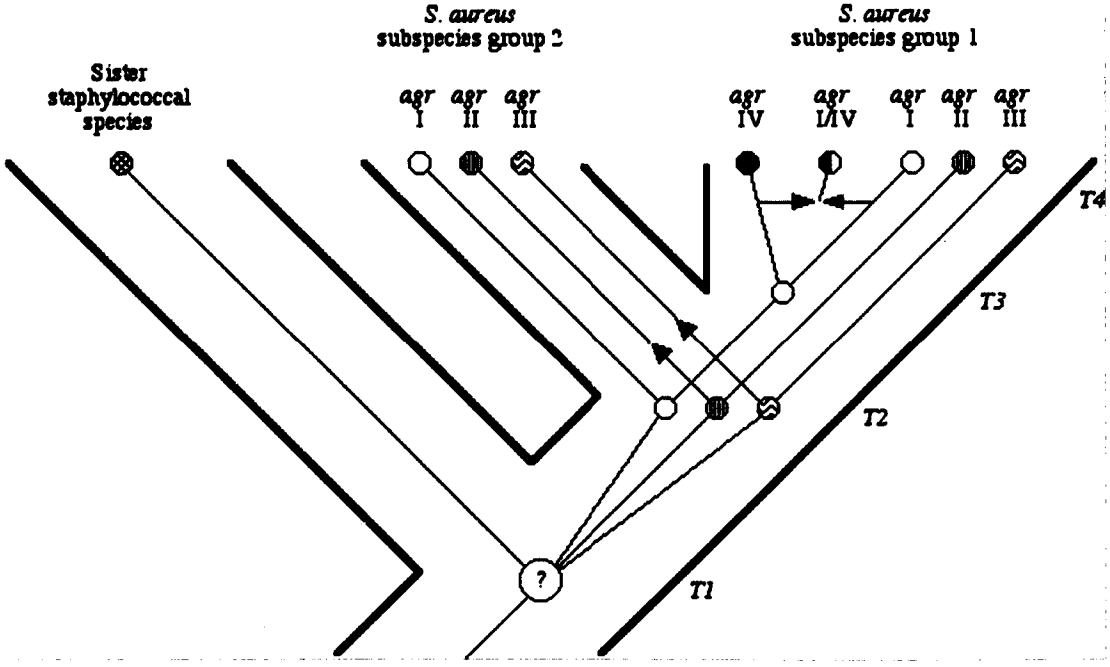



Figure 2.30 Model for the evolution of *agr*. The clone phylogeny (or species tree) is shown as a bold outline. The *agr* phylogenies are shown as thin lines within the clone phylogeny. Different *agr* groups are shown as shaded nodes[358].

As mentioned by Tenover *et al* no single typing method appears to be clearly superior in all cases[423]. Although, the current ability of *spa* typing to distinguish both molecularly and epidemiologically linked strains rapidly and easily makes it particularly well suited for the initial screening that may be used to identify and direct epidemiological studies.

### 2.4.2 Clones found among animal *S. aureus*

A subset of 49 animal isolates was selected and further genotyped by MLST and *spa* typing. This collection included 11 of the 13 animal sources, 15 of the 20 PF groups and 38 PF subtypes.

Fifty three isolates formed PF group A representing 14 PF subtypes. PFGE subtyping differentiated strains between different animal and geographic sources. The only exceptions to this were PF subtypes A7 and A12. PF subtype A7, the predominant subtype was detected in chicken and bovine in three different locations but not Highland and A12 was detected in pig and bovine from Grampian and Highland regions. Similar results have been reported by Zadoks *et al* and Annemüller *et al* where PFGE was able to differentiate *S. aureus* strains within and between bovine herds in the Netherlands and Germany[22,498]. Cluster analysis of these isolates was supported by MLST (CC5) and *spa* typing (*spa* CC2). The majority of isolates within PF group A were from avian sources including livestock and wild birds. Aires-de-Sousa *et al* have also reported the presence of *S. aureus* belonging to ST5 in bovine milk samples. In that study they suggest that this isolate, a genetic background mainly associated with cases of human disease, may have been the result of human contamination[15]. It is possible that the detection of ST5 strains, from cattle and pig sources from Grampian and Highland regions may be as a result of human contamination. In a study by Gilot and van Leeuwen they have shown that the transfer of *S. aureus* strains between humans and livestock animals although possible was an infrequent event[156]. The majority of isolates, however, within PF group A were isolated from multiple avian sources and geographic locations. This data casts doubt over the suggestion that these strains may have been as

a result of human contamination and indicate the possible presence of an avian adapted *S. aureus* clone.

Isolates belonging to ST9, *spa* CC1430 and PF group T, were found among cattle and pigs, suggesting that *S. aureus* from these animal sources do not represent separate genetic populations. These findings agree with those of Mørk *et al*, who genotyped *S. aureus* from cases of bovine, caprine and ovine mastitis in Norway. They found that a small number of closely related genotypes are responsible for the majority of cases of *S. aureus* mastitis and that these genotypes exhibit little or no host preference among these species[305]. A study by van Leeuwen *et al* reported that mastitis-related *S. aureus* from diverse hosts were genetically clustered suggesting tissue specificity not host specificity[452]. Contrasting with this conclusion is a study by Smith *et al* who reported that a single MLST CC (CC97) was responsible for the majority of cases of bovine mastitis. This CC, with ST97 as the predicted ancestor, comprised of 14 ST which included ST71 and ST25. These data are consistent with those of this study where all isolates belonging to CC97 were isolated from bovine sources. Unlike Smith *et al*, ST25 was not found to be associated with bovine species but companion animals in this study. ST25 has previously been identified as the ancestral clone of a CC and is a DLV of a DLV of ST97. This ST was placed as a satellite strain among CC97 with ST127 as an intermediary[401]. No intermediary strain was found between STs 25 and 97 in this study which explains why eBURST did not cluster these isolates. Although, an unrooted Bayesian tree produced by Feil *et al* supports the closer relationship of ST25 and ST97 than to other ancestral strains[132]. Aires-de-Sousa *et al* have also reported the isolation of isolates belonging to CC97 (ST747) from mastatic cattle in Brazil[15].

The minor MLST CC, CC814 contained two novel STs from strains of avian origin (chicken and pheasant) that were geographically separated. These isolates also belonged to two novel *spa* types (t1422 and new). Visual inspection of the unassigned new *spa* type, by the insertion of an additional r34 within the t1422 repeat succession, clearly links these isolates. The data from MLST and *spa* typing supports the clustering of these isolates within PF group M. It appears that this CC is divided across animal species and geographic lines.

PF group Q comprised of two subtypes, both of which belonged to ST133 and *spa* type t1428. No reports of human colonization or infection with this clone could be found although cases of bovine and caprine mastitis have been reported caused by ST133 (http://www.mlst.net).

According to the criteria proposed by Tenover *et al*[424] it must be emphasized that these criteria apply only to epidemiologically related isolates recovered within a short time frame (<3 months) and a narrow geographic distribution, this is underlined by the observation that isolates assigned to the same PF group could belong to a different ST. This was the case for two sets of STs in this part of the study. A single isolate of ST774 was assigned to the same PF group as isolates within CC97 and differed at four of the seven MLST loci. Also the isolates that formed PF group J, a single isolate of ST385 and two isolates of ST816 differed at all seven loci. Melles *et al* in a study comparing MLST, PFGE and amplified fragment length polymorphism (AFLP), for typing *S. aureus*, have also shown a small number of isolates assigned to the same PFGE group that belonged to different CC[293]. Jørgensen *et al* have also shown that isolates, from bovine bulk milk, assigned to the same PF group belonged to different STs that differed

from each other at all seven loci[211]. Also, isolates that belong to identical STs could also belong to different PF groups. Isolates within PF groups A and C both belonged to ST5 and *spa* CC2 and isolates belonging to PF groups D, G, P and U all belonged to MLST CC97. Melles *et al* concluded that PFGE, which does not include an amplification step, is a less useful method for long term epidemiological surveillance[293].

### 2.4.3 Genotypic comparison of human and animal *S. aureus* strains

Twenty eight human *S. aureus* were selected for genotypic characterization and comparison with animal strains based on their RS-PCR and PFGE banding pattern similarity to those found among animal strains.

Six STs were common to both animal and human isolates with four of these STs (ST5, ST22, ST30 and ST45) representing four of the five major contemporary epidemic MRSA clonal lineages. Cluster data obtained by MLST, *spa* typing, PFGE analysis and *agr* typing demonstrated a high degree of congruence. Koreen *et al* has also shown a high level of congruence between genotyping methods and concluded that this indicated a low level of recombination, a predominantly clonal population structure, and the presence of a phylogenetic signal within *S. aureus*[239]. Similar results have been reported for clonal complexes containing major MSSA and epidemic MRSA isolates, such as CC5, CC8, CC30 and CC45[98,239]. Although, *agr* typing has been shown to be a more variable characteristic in some studies[98].

Isolates belonging to CC5 corresponded to *spa* CC2, containing four different *spa* types and *agr* type II and PF type 105. ST5 is an old and globally spread lineage which is the predicted founder of CC5 and is the genetic background to the New York/paediatric

MRSA clone and represents most of the MRSA with reduced susceptibility to vancomycin[404].

Four isolates, two animal and two human, were ST22, *agr* type I with EMRSA15 patterns. These isolates also belonged to *spa* CC223 containing two *spa* types (t223 both animal isolates and t1433 both human isolates). The *spa* types within this CC clustered at a calculated cost of ≤1 indicating a closely related clonal lineage. This clone rapidly emerged and spread during the 1990s and is today widely disseminated in hospitals in the UK (EMRSA 15) and in Germany (Barnim epidemic MRSA)[153]. ST22 MRSA has also been isolated from companion animal derived infections that belong to the same clonal lineage as those found among the human population, in hospitals and the community, and represent a transmission risk to human handlers[270,301].

Isolates belonging to ST25 have been reported, together with ST1 and 30, as a significant cause of community acquired disease in humans and has also been reported as the cause of disease among bovine sources[122,400]. Smith *et al* and Feil *et al* have both suggested a closer relationship between ST25 and ST97 than to other ancestral strains[132]. With the exception of *agr* typing, none of the genotypic methods used in this study linked these clonal backgrounds.

Four isolates of ST30, three human and one companion animal, were detected. This clonal background has been identified as a successful internationally distributed MSSA lineage, associated with invasive disease in humans and has been isolated from bovine milk and animal bedding samples from the USA[401]. ST30 is closely related to the EMRSA16 pandemic clone (ST36) responsible, together with EMRSA15 (ST22), for 95% of MRSA bacteraemia in the UK[14,19,122].

A study characterizing toxic shock syndrome toxin 1 positive MSSA from Germany has the presence of isolates belonging to ST45, the genotypic background to the Berlin MRSA clone[249]. These isolates belonged to *spa* and *agr* types common to the human isolates of ST45 detected in this study. Two animal isolates of ST47, a SLV of ST45, belonged to the same *agr* type as the human strains. As the animal isolates had less than five *spa* repeats in the sequence these were excluded from the BURP analysis, although visual inspection shows them to be closely related to the human strain *spa* types.

In conclusion, this study has shown that PFGE, MLST and *spa* typing can be successfully applied to characterize *S. aureus* isolated from animals. MLST and *spa* typing data were easier to interpret than banding patterns generated by PFGE. *spa* typing was superior in discriminatory power than both MLST and PFGE. Combining *spa* and MLST increased the number of types found and the discriminatory power although the routine use of both methods would be prohibitively expensive. Therefore, *spa* typing represents a suitable single genotyping technique for the characterization of *S. aureus* isolated from animals.

A large number of *S. aureus* types were found among the animal isolates, but four types were predominant and were very closely associated to an animal source and geographic region.

Six of the observed STs were common to the animal and human isolate collections. These STs have previously been reported from cases of human disease and from healthy carriers. The presence of isolates assigned to these STs in animals could reflect human contamination. This is, however, not conclusive as many of these strains have been reported previously in cases of animal infection[136,211].

## Chapter 3 Host specificity

### 3.1 Introduction

*S. aureus* can colonise and infect a variety of members of the animal kingdom, including mammals, reptiles, and birds[452]. Studies have addressed the molecular basis of host specificity among *S. aureus* strains. These have examined gene polymorphisms with multilocus enzyme electrophoresis, PFGE, binary typing and shotgun sequencing and have shown that *S. aureus* strains from humans can and do cross-infect domestic and companion animals[270,467-469,497,498]. The concept of host specificity among bacterial genera is not new. For example, *S. enterica* serotypes *Dublin* and *Rostock* are known to be host specific for bovine and *Choleraesuis* for swine[442]. Willems *et al* have shown host specificity in *E. faecium* strains based on amplified fragment length polymorphism analysis of vancomycin resistant strains from humans and a range of domestic and companion animals[483]. Devriese *et al* have also concluded that *E. faecium*, with different carbohydrate requirements, were associated with different host species and suggested that certain enterococci are host specific[102].

Most of the work examining the possibility of host specificity of *S. aureus* strains has been performed on isolates from cases of bovine mastitis. Numerous methods have been used, including phenotypic, phage typing and antibiogram, genotypic, determining gene polymorphisms with MLEE, PFGE and binary typing, MLST and *spa* typing and the detection of specific virulence factors, such as toxins, adhesins and global regulators, using molecular techniques[63,299,305,370,398,401,452,497,498]. Although some strains identified in these studies were closely related to isolates recovered from humans, many were not, and it has been suggested that the successful transfer of *S. aureus* between humans and

domestic animals under natural conditions is limited. Reports of *S. aureus* from companion animals, as a source of infection in humans, are becoming more frequent[270,466,467]. The molecular basis of host specificity in *S. aureus* remains unknown[183].

The purpose of this study was to determine the genetic relationship between *S. aureus* isolates from animals and human by examining a number of recognized virulence determinants.

## 3.2 Materials and methods

### 3.2.1 Bacterial strains

Two strain collections were used; the first strain collection comprised 49 animal strains (Table 2.19), the second strain collection consisted of 28 human *S. aureus* strains (Table 2. 24). Both collections were characterized to clonal level in chapter 2.

### 3.2.2 Virulence factor PCR

For both strain collections, the genes coding for adhesins, the global regulator *agr*, staphylocoagulase, enterotoxins, haemolysins and exoprotein toxins were amplified using the PCR primers and amplification conditions outlined in Table 3.1 and Table 3.2. PCR amplification was performed on a Techne Genius thermal cycler. A positive and negative control was included in each PCR run.

Table 3.1 PCR reaction mixes

| PCR reaction mixture | Template DNA[a] | Primer sequence[b] | PCR mastermix[c] | Ready-to-go bead reaction tube[d] | Tissue grade water[e] | Total volume |
|---|---|---|---|---|---|---|
| PCR-1 | 1µl | 1µl | 12.5µl | | 8.5µl | 23µl |
| PCR-2 | 1µl | 1µl | | 1 | 21µl | |
| multiplex PCR | 2µl | 2µl | | 1 | 21µl | 25µl |

[a]Template DNA was extracted as described in section 2.5.1

[b]Primer sequences- PCR reaction mixture 1 and 2, 0.5µl of each forward and reverse primer and multiplex PCR, 0.5µl of each forward and reverse primer added to a microtube. 2µl of primer mix used in multiplex PCR. All primers were prepared at 50pmol concentration.

[c]Reddymix PCR mastermix (ABgene, UK) (0.2 mM of dATP, dCTP, dGTP and dTTP, 3 mM $MgCl_2$, 0.625 units Taq polymerase, 20mM Trizema base and 50 mM

[d]Supplied by Amersham pharmacia, UK

[e]Supplied by Sigma, UK

Table 3.2 PCR primers and conditions for identification of potential virulence genes

| Primer | Product (bp) | Primer sequence 5'-3' | Positive control | Negative control | PCR reaction mix and conditions | Reference |
|---|---|---|---|---|---|---|
| *sea*-1 | 120 | TTGGAAACGGTTAAAACGAA | NCTC 10651 | Tissue grade water | Multiplex PCR.<br>Initial denature 95°C 4 minutes, 1 cycle of 95°C 4 minutes, 30 cycles of 95°C 30 seconds, 55°C 30 seconds, and 72°C 1 minute, final extension 72°C 5 minutes | Becker *et al*[33,34], Brakstad *et al*[52], Johnson *et al*[207], Sakurai *et al*[366] and Lina *et al*[262] |
| *sea*-2 | | GAACCTTCCCATCAAAAACA | | | | |
| *seb*-3 | 163 | GTATGGTGGTGTAACTGAGC | | | | |
| *seb*-4 | | CCAAATAGTGACGAGTTAGG | | | | |
| *sec*-3 | 271 | CTCAAGAACTAGACATAAAAGCTAGG | NCTC 11963 | | | |
| *sec*-4 | | TCAAAATCGGATTAACATTATCC | | | | |
| *sed*-3 | 319 | CTAGTTTGGTAATATCTCCTTTAAACG | NCTC 10656 | | | |
| *sed*-4 | | TTAATGCTATATCTTATAGGGTAAACATC | | | | |
| *see*-3 | 178 | CAGTACCTATAGATAAAGTTAAAACAAGC | FRI 326 | | | |
| *see*-2 | | TAACTTACCGTGGACCCTTC | | | | |
| *tst*-3 | 445 | AAGCCCTTTGTTGCTTGCG | NCTC 11963 | | | |
| *tst*-6 | | ATCGAACTTTGGCCCATACTTT | | | | |
| *eta*-1 | 741 | CTATTTACTGTAGGAGCTAG | eta | | | |
| *eta*-2 | | ATTTATTTGATGCTCTCTAT | | | | |
| *etb*-1 | 629 | ATACACACATTACGGATAAT | etb | | | |
| *etb*-2 | | CAAAGTGTCTCCAAAAGTAT | | | | |
| *luk-pv*-1 | 433 | ATCATTAGGTAAAATGTCTGGACATGATCCA | Mulvey | | | |
| *luk-pv*-2 | | GCATCAAATGTATTGGATAGCAAAAGC | | | | |
| *nuc*-1 | 280 | GCGATTGATGGTGATACGGTT | | | | |
| *nuc*-2 | | AGCCAAGCCTTGACGAACTAAAGC | | | | |
| *icaA*-1 | 770 | GATTATGTAATGTGCTTGGA | EMRSA16 | | PCR-1.<br>Initial denature 94°C 4 minutes, 30 cycles of 94°C 1 minute, 50°C 1 minute, and 72°C 1 minute, final extension 72°C 4min | Crampton *et al*[84] |
| *icaA*-2 | | ACTACTGCTGCGTTAATAAT | | | | |

Table 3.2 PCR primers and conditions for identification of potential virulence genes

| Primer | Product (bp) | | Positive control | Negative control | PCR reaction mix and conditions | Reference |
|---|---|---|---|---|---|---|
| *fnbA*-1 | 1,362 | CACAACCAGCAAATATAG | NCTC 8325-4 | | PCR-2.<br><br>Initial denature 94°C 4 minutes, 30 cycles of 94°C 1 minute, 50°C 1 minute, and 72°C 2 minutes, final extension 72°C 4 minutes | Peacock *et al*[332] |
| *fnbA*-2 | | CTGTGTGGTAATCAATGTC | | | | |
| B1 | 1,070 and/or c650 | TATGCTCCTGCAGCAACTAA | * | Tissue grade water | PCR-2<br><br>initial denature 94°C 5 minutes, 40 cycles of 94°C 1 minute, 50°C 2 minutes, and 74°C 3 minutes, final extension 74°C 3 minutes | Gilot *et al*[156] |
| C2 | | CTTGCGCATTTCGTTGTTGA | | | | |
| COAG-1 | 1.4kb | CAGATGCGATAGTAACAAAGGATT | NCTC 8325 | NCTC 11047 | PCR-1<br><br>Initial denature 94°C 4 minutes, 30 cycles of 94°C 1 minute, 51°C 1 minute, and 72°C 2 minutes, final extension 72°C 4 minutes | Carter et al[63] |
| COAG-4 | | TGTTCCATCGTTGTATTCAC | | | | |
| COAG5 | multiples of 81bp + 18bp terminal sequence | TATGAAGCGAGACCAAGATT | | | | |
| COAG-6 | | TGTCGCAGTACCATCTGC | | | | |
| HLA-1 | 209 | CTGATTACTATCCAAGAAATTCGATTG | 04.7486.G | Tissue grade water | Multiplex PCR<br><br>Initial denature 94°C 4 minutes, 1 cycle 95°C 4 minutes, 30 cycles of 95°C 30 seconds, 55°C 30 seconds, and 72°C 1 minute, final extension 72°C 5 minutes | Jarraud *et al*[201] |
| HLA-2 | | CTTTCCAGCCTACTTTTTTATCAGT | | | | |
| HLB-1 | 309 | GTGCACTTACTGACAATAGTGC | | | | |
| HLB-2 | | GTTGATGAGTAGCTACCTTCAGT | | | | |
| HLG-1 | 950 | GCCAATCCGTTATTAGAAAATGC | | | | |
| HLG-2 | | CCATAGACGTAGCCACGGAT | | | | |
| HLD-1 | 111 | AAGAATTTTTATCTTAATTAAGGAAGGAGTG | | | | |
| HLD-2 | | TTAGTGAATTTGTTCACTGTGTCGA | | | | |
| cap5-1 | 881 | ATGACGATGAGGATAGCG | Reynolds strain | | PCR-1<br><br>Initial denature 94°C 3 minutes, 32 cycles of 94°C 30 seconds, 55°C 30 seconds, and 72°C 1 minute, final extension 72°C 4 minutes | Moore *et al*[302] |
| cap5-2 | | CTCGGATAACACCTGTTGC | | | | |
| cap8-1 | 1148 | ATGACGATGAGGATAGCG | EMRSA16 | | | |
| cap8-2 | | CACCTAACATAAGGCAAG | | | | |

* No control strain required

### 3.2.2.1 Gel electrophoresis

Electrophoresis was performed using six microlitres of PCR product loaded in a 1.5% Neuseive agarose (Cambrex, UK) gel in a 0.5% TBE solution (Gibco). Staphylococcal enterotoxin genes were seperated at 180V for 210 minutes and all other virulence factor genes were separated at 140V for 90 minutes. Following electrophoresis gels were stained with 1μg/mL ethidium bromide solution (Sigma, UK) for 30 minutes visualised by uv transillumination (UVP, UK) and the image stored digitally using VisionWorks 32 software. The size of each PCR product was determined by comparison with the appropriate control strain and molecular weight ladder (Gibco, UK).

### 3.2.2.2 Restriction endonuclease digestion

Endonuclease digest reactions were performed in a 20μl sample containing 12μl of tissue grade water (Sigma,UK), 5μl of PCR product, 2μl of appropriate REact® buffer and 1μl of restriction endonuclease. Digest products were analysed by gel electrophoresis using the method outlined in section 3.2.2.1.

### 3.2.3 Biofilm assay

The production of biofilm was detected following the method of Vasudevan *et al*[455]. Three colonies of each test isolate was added to a 2ml tryptone soya broth containing 0.25% (w/v) glucose (TSBG) and incubated overnight at 37°C. Following incubation 5μl of overnight broth suspension was added to 195μl of sterile TSBG in a "U" bottom polystyrene tissue culture well (Nunc GmbH, Germany). Cultures were incubated overnight at 37°C followed by three washes in phosphate buffered saline (PBS). Each well was stained for 5 minutes using

200µl of 1% crystal violet, washed three times with PBS, and dried at 37°C. The absorbance of each well was determined at 570 nm (Titrec plate reader). A positive control, *S. epidermidis* (ATCC 35984), negative control, *S. epidermidis* (ATCC 12228), and three wells containing only TSBG were included in each biofilm assay. The mean absorbance value for the wells containing only TSBG was subtracted from each unknown absorbance to correct for background effects. Strains were considered biofilm positive if the corrected absorbance value was ≥0.1. Biofilm assays were performed in triplicate.

### 3.2.4 Statistical analysis

The distribution of virulence factors from animal and human *S. aureus* strains were compared using Fisher's exact test. *P* values of less than 0.05 were considered significant[452].

## 3.3 Results

### 3.3.1 Distribution of virulence factors

#### 3.3.1.1 *agr* RFLP

The 1,070-bp variable region of the *agr* operon that includes AgrD, the gene responsible for the group specific autoinducing peptide, was amplified by PCR with primers B1 and C2 (Figure 3.1). Among the 77 *S. aureus* isolates investigated 54 (70.1%) of the strains tested produced two amplicons of 1,070bp and approximately 650bp (type A), 20 (26%) isolates produced an amplicon of 1,070bp (type B), two isolates produced an amplicon of approximately 650bp (type C), and no amplicon was produced from a single isolate (type D) (Figure 3.2).

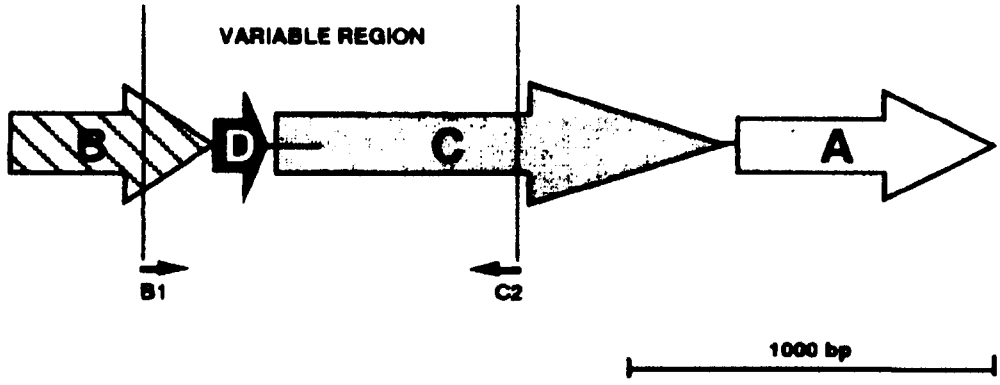



Figure 3.1 Schematic map of the *S. aureus* agr operon. *agrA* and *agrC* encode the response regulator and receptor histidine protein kinase components of a two-component signal transduction pathway, whereas *agrB* and *agrD* combine to generate the activating ligand for the receptor. Small arrows indicate position

of the B1 and C2 primers used to amplify the variable region of the agr operon[156]

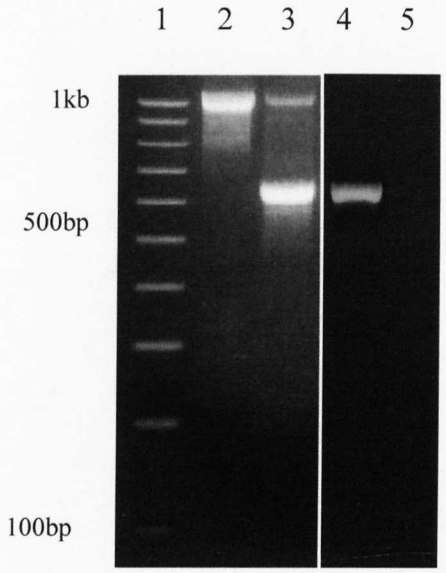
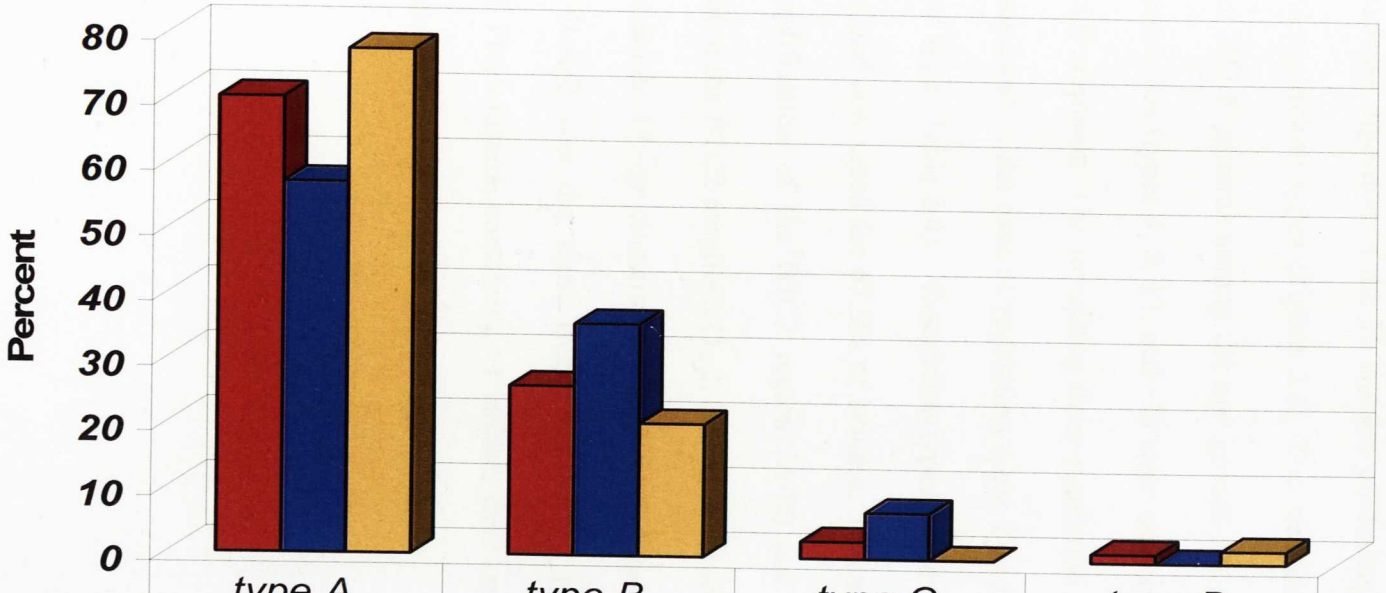



Figure 3.2 Presentation of *agr* B1C2 PCR products. Lane 1, 100bp molecular weight ladder; Lane 2-5 correspond to isolates 01.9732.Y (type B), 02.4275.G (type A), 04.1039.F (type C) and 02.4290.M (type D)

A variation in the distribution of B1C2 amplicons was detected between the animal and human *S. aureus* strains (Figure 3.3). Isolates within the animal strain collection exhibited a higher frequency of type A amplicons, while isolates from the human strain collection exhibited a higher frequency of type B amplicons. The 650bp type C amplicon was detected only from human strains. No amplicon (type D) was detected from a single animal isolate (Figure 3.3).

Figure 3.3 Distribution of *agr* B1C2 amplicons



Polymorphism of the *agr* locus was determined by *Alu*I restriction profile analysis after amplification of the variable region of the *agr* operon. Endonuclease digestion of the 76 isolates producing an amplicon yielded eight different restriction types (Figure 3.4). The frequency and distribution of the different RFLP patterns among the *agr* groups are outlined in Table 3.4. Four restriction types (types 4, 8, 12, and 13) were associated with single *agr* groups and B1C2 amplicon. The remaining three restriction types belonged to multiple *agr* groups and in the case of restriction types 2 and 3 to more than one B1C2 amplicon type (Table 3.4). Restriction types 2 and 3 were the most frequently detected and accounted for 67.5% of isolates. Taken together, *agr* grouping (I-IV), amplification of the B1C2 region (A-D) and subsequent endonuclease digestion of the B1C2 amplicon (1, 2, 3, 4, 6, 8, 12 and 13), *agr* typing grouped all isolates into 18 *agr* clusters, of which seven were single isolate (Table 3.4). Cluster II-A-2 was the most frequent (31.2%), followed by cluster I-A-3 (27.3%). Five clusters, containing >1 isolate, were represented by either animal or human strains only.

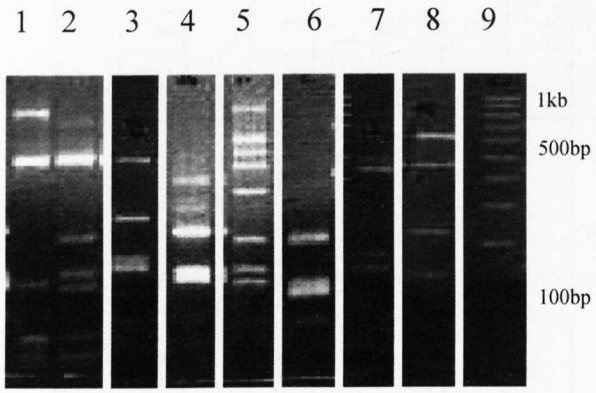



Figure 3.4 Restriction types of *agr* B1C2 PCR amplicons. Lanes numberd 1-9 correspond to isolates in table 3.3.

Table 3.3 Isolates represented in Figure 3.4

| Lane | Isolate number | Restriction type | ST | Isolate source |
|---|---|---|---|---|
| 1 | 02.4255.S | 1 | 22 | Milk |
| 2 | 01.9706.Z | 2 | 5 | Chicken |
| 3 | 02.7568.H | 3 | 25 | Human |
| 4 | 02.4297.H | 4 | 47 | Cow |
| 5 | 01.9747.H | 6 | 30 | Horse |
| 6 | 01.9771.Y | 8 | 22 | Pig |
| 7 | 02.2794.S | 12 | 133 | Lamb |
| 8 | 01.9798.L | 13 | 816 | Horse |
| 9 | 100bp molecular weight ladder | | | |

Table 3.4 Distribution of *agr* group, B1C2 amplicon and restriction type

| *agr* group | B1C2 amplicon | B1C2 restriction type | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 6 | 8 | 12 | 13 |
| I | A | 3[a] | | 19 | | | | 2[a] | |
| | B | 5[a] | | 3 | 2[a] | | 5 | | |
| II | A | | 20 | 5 | | 1 | | | 1 |
| | B | | 2[h] | | | | | | |
| | C | | 1 | | | | | | |
| III | A | 1 | | | | 1 | | | |
| | B | | | | | 3 | | | |
| | C | | 1 | | | | | | |
| IV | A | | 1 | | | | | | |
| Total | | 9 | 26 | 26 | 2 | 4 | 6 | 2 | 1 |

[a]clusters containing strains of animal origin only

[h]clusters containing strains of human origin only

Of the five STs common to the animal and human isolate collections four were placed within the same *agr* cluster (Table 3.5). Isolates belonging to the remaining ST, ST15, were placed within different agr clusters. ST45 (human strain) and ST47 (animal strain), a SLV of ST45, although represented within the same *agr*-B1C2 type were separated into different *agr* clusters following restriction analysis.

Table 3.5 *agr* clusters and associated STs

| *agr* cluster | Source | Associated STs |
|---|---|---|
| I-A-3 | Animal | **25**, 71, 97, 188, 692, 814, 815 |
| | Human | 8, 20, **25**, 789 |
| I-B-3 | Animal | 71 |
| | Human | **25**, 47 |
| I-B-8 | Animal | **22** |
| | Human | **22**, 45 |
| II-A-2 | Animal | **5**, 9 |
| | Human | **5**, **15**, 109 |
| II-A-3 | Animal | **5**, 385, 816 |
| | Human | 49, **125** |
| II-A-6 | Animal | **15** |
| II-B-6 | Animal | **30** |
| | Human | **30** |

### 3.3.1.2 Exotoxin PCR

The presence of five staphylococcal enterotoxin genes, *sea*, *seb*, *sec*, *sed*, and *see* and five exoprotein toxin genes, *eta*, *etb*, *lukS-pv*, *lukF-pv* and *tst* was determined for the two strain collections (Figures 3.6 and 3.7). Among the 77 *S. aureus* isolates investigated, 28 (36.4%) isolates carried one or two enterotoxin genes. Seven isolates

(9.1%) (one animal and six human) contained the gene encoding SEA, eight isolates (10.4%) (four animal and four human) contained the gene encoding SEC, and thirteen isolates (16.9%) (ten animal and three human) contained the gene encoding TSST-1 (Figure 3.7). Four of the seven isolates containing SEA genes were simultaneously positive for TSST-1, and one of the eight isolates containing SEC was also positive for TSST-1. None of the 77 isolates harboured the genes encoding SEB, SED, SEE, ETA, ETB or PVL.

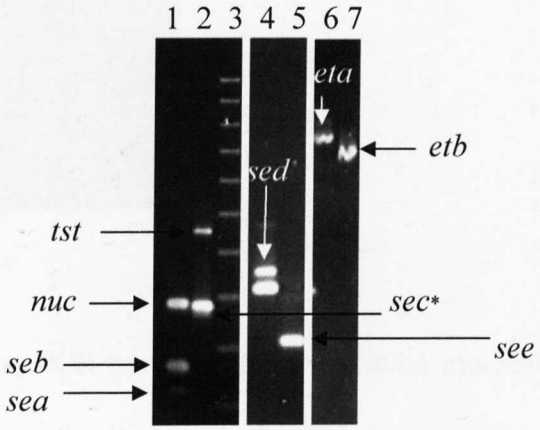



Figure 3.5 Presentation of the genes encoding staphylococcal enterotoxin A, B, C, D and E and epidermolytic toxins A and B. Nuclease gene (*nuc*) used as an internal control. Lanes 1-7 correspond to isolates in table 3.6. * sec is a weak band with only a nine base pair difference from the strong *nuc* band.

Table 3.6 Isolates represented in Figure 3.5

| Lane | Toxin gene | Amplicon size (bp) | Isolate |
|---|---|---|---|
| 1 | *sea* and *seb* | 120, 163 | *S. aureus* NCTC 10615 |
| 2 | *sec* and *tst* | 271, 445 | *S. aureus* NCTC 11963 |
| 3 | 100bp molecular weight ladder | | |
| 4 | *sed* | 319 | *S. aureus* NCTC 10656 |
| 5 | *see* | 178 | FRI 328 |
| 6 | *eta* | 741 | eta |
| 7 | *etb* | 629 | etb |

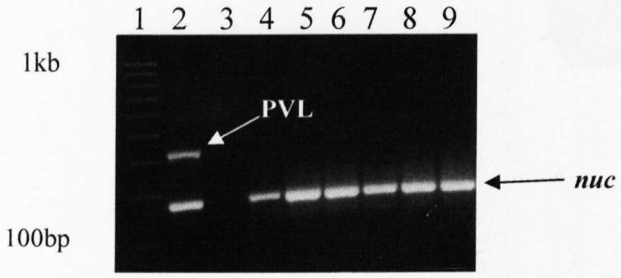
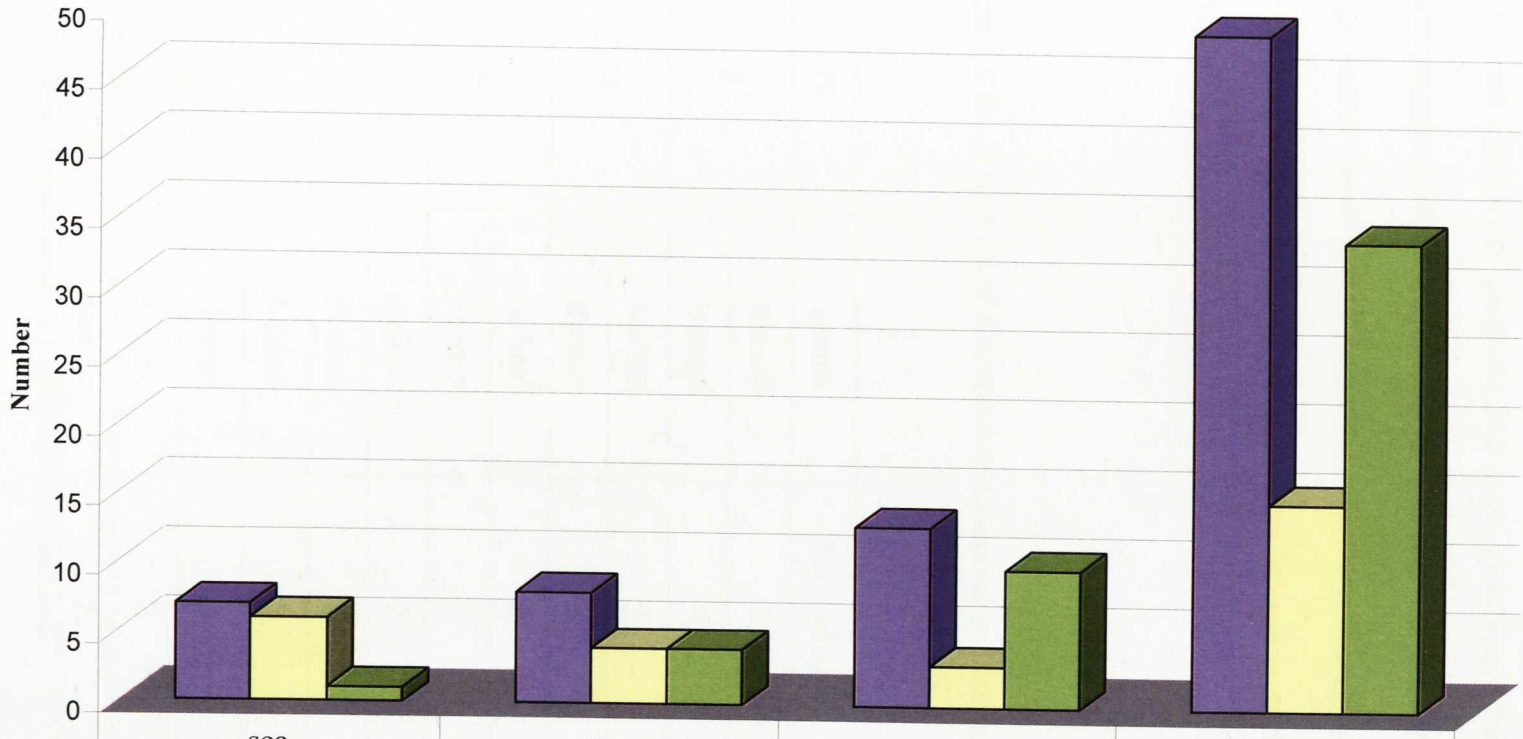



Figure 3.6 PVL gene PCR products; Lane 1, 100bp molecular weight ladder; Lane 2, Mulvey positive control strain; Lane 3, Water control; Lanes 4-9, correspond to isolates in Table 3.7.

Table 3.7 Isolates represented in Figure 3.6

| Lane | Isolate | Animal source | ST |
|---|---|---|---|
| 4 | 01.9706.Z | Chicken | 5 |
| 5 | 02.4275.G | Cow | 15 |
| 6 | 01.9771.Y | Pig | 22 |
| 7 | 01.9761.B | Parrot | 25 |
| 8 | 01.9747.H | Horse | 30 |
| 9 | 02.2782.Y | Unknown | 47 |

Figure 3.7 Distribution of toxin genes *sea*, *sec* and *tst*




| | sea | sec | tst | negative |
|---|---|---|---|---|
| Total (n=77) | 7 | 8 | 13 | 49 |
| human (n=28) | 6 | 4 | 3 | 15 |
| animal (n=49) | 1 | 4 | 10 | 34 |

Eight of the 27 isolates (16 animal and 11 human), representing the STs common to both strain collections, produced four toxin genotypes (Table 3.8). The *sea* gene was overrepresented within the human strain collection (Fisher's exact test; $P$=0.027). A single animal isolate of ST5 carried *tst*, all isolates belonging to ST15 were negative for the presence of toxin genes, human isolates belonging to ST22 and ST25 carried *sea* and animal and human isolates of ST30 carried *sea* and *tst*.

Table 3.8 Toxin profiles of STs common to animal and human strain collections

| ST | source | No. isolates | toxin profile |
|---|---|---|---|
| 5 | Animal | 7 | *tst* (1) |
| | Human | 4 | Negative |
| 15 | Animal | 1 | Negative |
| | Human | 3 | Negative |
| 22 | Animal | 2 | Negative |
| | Human | 2 | *sec* (2) |
| 25 | Animal | 2 | Negative |
| | Human | 2 | *sea* (1) |
| 30 | Animal | 1 | *sea*+*tst* (1) |
| | Human | 3 | *sea*+*tst* (2), *tst* (1) |
| ST45/ST47 | Animal | 2 | *sec* (2) |
| | Human | 2 | *sec* (2) |

The number of isolates that tested positive for a toxin gene is given in parentheses

### 3.3.1.3 *icaA* and biofilm production

Seventy three (94.8%) isolates were positive for the presence *icaA*, responsible for encoding polysaccharide intercellular adhesin (Figure 3.8). The four negative isolates were all of bovine origin.

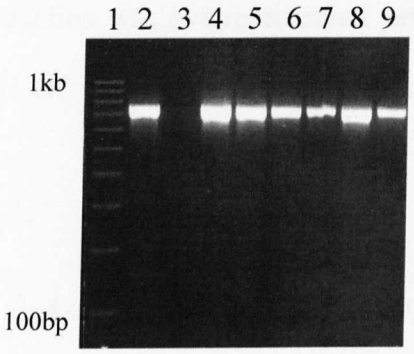



Figure 3.8 *icaA* gene PCR products. Lane 1, 100bp molecular weight ladder; Lane 2, EMRSA 16 positive control strain; Lane 3, Water control; Lanes 4-9, correspond to isolates in Table 3.9. The difference in band thickness in lanes 7 and 9 is due to a difference in the amount of DNA produced during PCR.

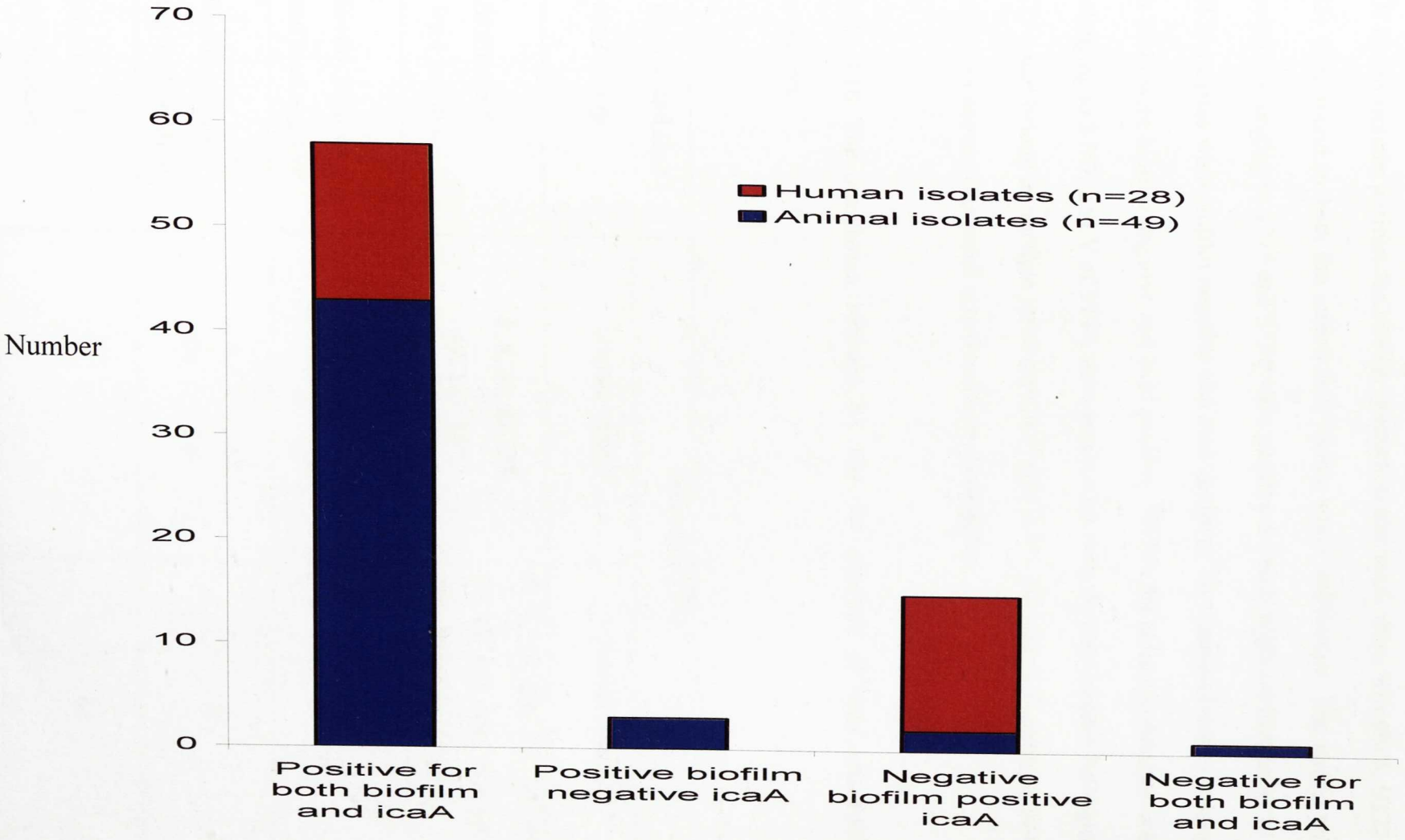
Table 3.9 Isolates represented in Figure 3.8

| Lane | Isolate | Animal source | ST |
|---|---|---|---|
| 4 | 01.9706.Z | Chicken | 5 |
| 5 | 02.4275.G | Cow | 15 |
| 6 | 01.9771.Y | Pig | 22 |
| 7 | 01.9744.S | Horse | 25 |
| 8 | 01.9747.H | Horse | 30 |
| 9 | 01.4297.H | Cow | 47 |

Of the strains tested 79.2% produced $A_{570}$ >0.1 (corrected for medium background) and were considered biofilm formers. Only three animal isolates were negative for biofilm production and belonged to ST5 and ST97. In contrast, 13 (46%) of human strains were negative and belonged to one of eight STs (ST1, ST8, ST15, ST30, ST45, ST109, ST123 and ST789) three of which were common to both isolate collections. Therefore, biofilm production was overrepresented among the animal strains (Fisher's exact test; $P$=<0.0001). Of the remaining two common STs both were positive for biofilm production.

Combining *icaA* and biofilm production data generated four different result outcomes. Fifty eight isolates were positive for both, three animal isolates were biofilm positive but negative for *icaA*, 46.4% of the human strains were negative for biofilm production and positive for *icaA* and a single animal isolate was negative for both (Figure 3.9).

Figure 3.9 Distribution of the *icaA* gene and biofilm production



Of the 58 isolates positive for biofilm production and *icaA*, three STs (ST5, ST22 and 25) were found in both the animal and human strain collections. The single animal isolates belonging to ST15 and ST30 were positive for both while all human ST15 and ST30 isolates were biofilm negative and *icaA* positive. Two human isolates belonging to ST45 were biofilm negative and *icaA* positive. Within the animal collection isolates belonging to ST47, a SLV of ST45, were positive for both. Animal isolates belonging to ST5 could belong to multiple result groups (Table 3.10). No other ST common to both isolate collections was found across multiple result groups.

Table 3.10 The association between ST and the presence of *icaA* and biofilm production.

| Biofilm and *icaA* result group | Associated STs | |
|---|---|---|
| | Human strains | Animal strains |
| Biofilm and *icaA* positive | **5**, 8, 20, **22**, **25**, 49, 125, 247, | **5**, 9, 15, **22**, **25**, 30, 47, 71, 97, 133, 188, 385, 692, 773, 774, 814, 815, 816 |
| Biofim positive and *icaA* negative | | 5, 71 |
| Biofilm negative and *icaA* positive | 1, 8, 15, 30, 45, 109, 123, 789 | 5 |
| Biofilm and *icaA* negative | | 97 |

### 3.3.1.4 *fnbA* PCR

Fifty (64.9%) of the 77 isolates were positive for the *fnbA* gene, responsible for encoding a cell wall adhesin (Figure 3.10). The *fnbA* gene was overrepresented within the human isolates ($P$=<0.0001) (Figure 3.11).

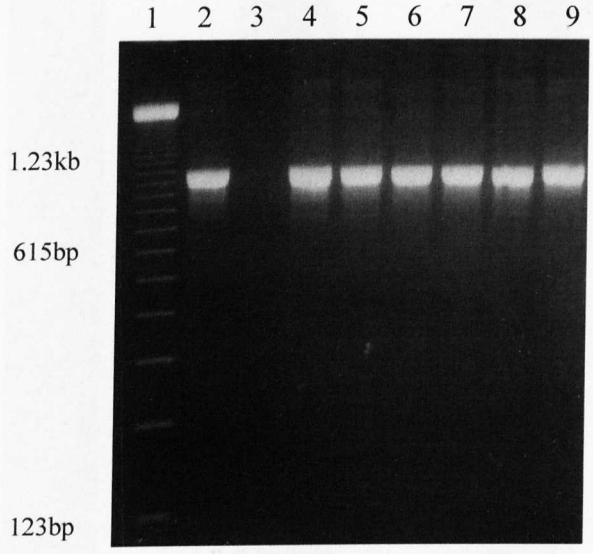
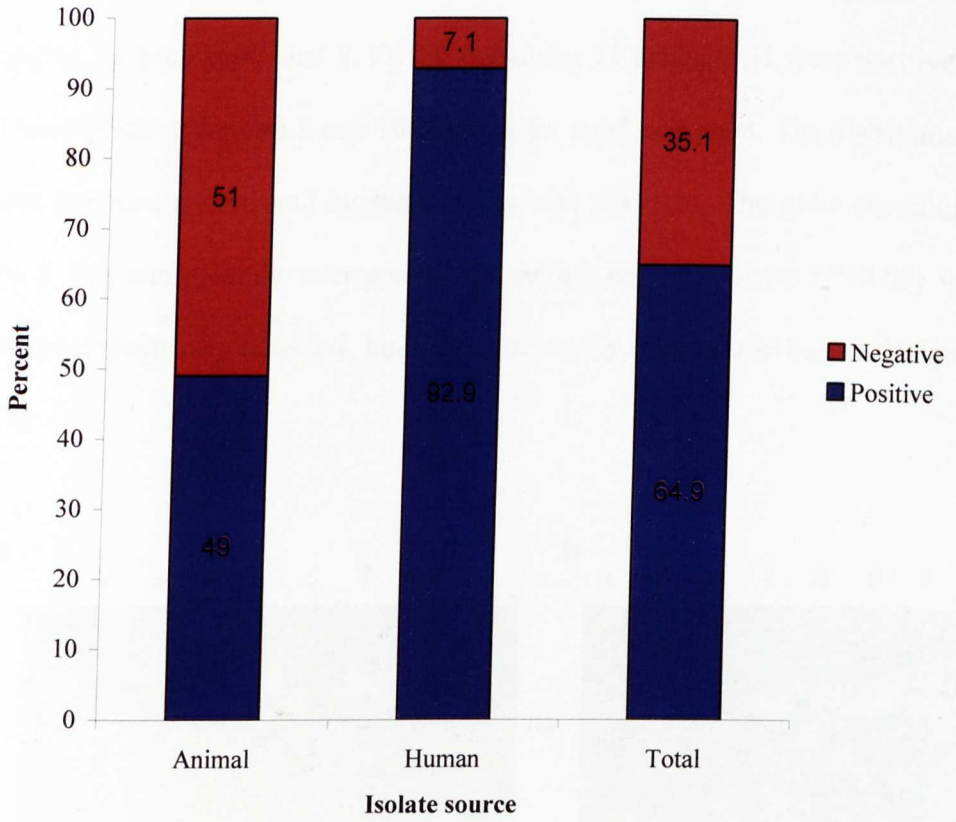



Figure 3.10 Presentation of *fnbA* gene PCR products. Lane 1, 123bp molecular weight ladder; Lane 2, *S. aureus* NCTC 8325-4 positive control; Lane 3, water control; Lanes 4-9 correspond to isolates in Table 3.11.

Table 3.11 Isolates represented in Figure 3.10

| Lane | Isolate | Animal source | ST |
|---|---|---|---|
| 4 | 01.9796.B | Phillips goose hawk | 5 |
| 5 | 02.4275.G | Cow | 15 |
| 6 | 01.9771.Y | Pig | 22 |
| 7 | 01.9761.B | Parrot | 25 |
| 8 | 05.1058.T | Human | 30 |
| 9 | 02.4297.H | Cow | 47 |

Figure 3.11 Distribution of the *fnbA* gene among *S. aureus* isolates from animal and human sources




Of the STs common to both isolate collections those belonging to ST15, ST22 and ST45/ST47 were positive for *fnbA* (Table 3.12).

Table 3.12 The association between ST and the presence of *fnbA*

| ST | Animal isolates | Human isolates |
|---|---|---|
| 5 | +/- | + |
| 15 | + | + |
| 22 | + | + |
| 25 | +/- | + |
| 30 | - | +/- |
| 45/47 | + | + |

### 3.3.1.5 *cap*5 and *cap*8 PCR

PCR amplification of the genes encoding capsular type 5 (*cap* 5) and 8 (*cap* 8) allowed the detection of these genes in 92.2% of isolates (Figure 3.12 a and b). Six isolates were negative for both *cap* 5 and 8. Of the remaining 71 isolates, 31 were positive for *cap* 5, 30 were positive for *cap* 8 and 10 positive for *cap*5 and *cap*8. The distribution of these genes between animal and human isolates was not even. The gene encoding capsular type 5 was significantly overrepresented among animal isolates ($P$=0.05) while *cap*8 was most frequently detected, but not significantly, among the human isolates (Figure 3.12).

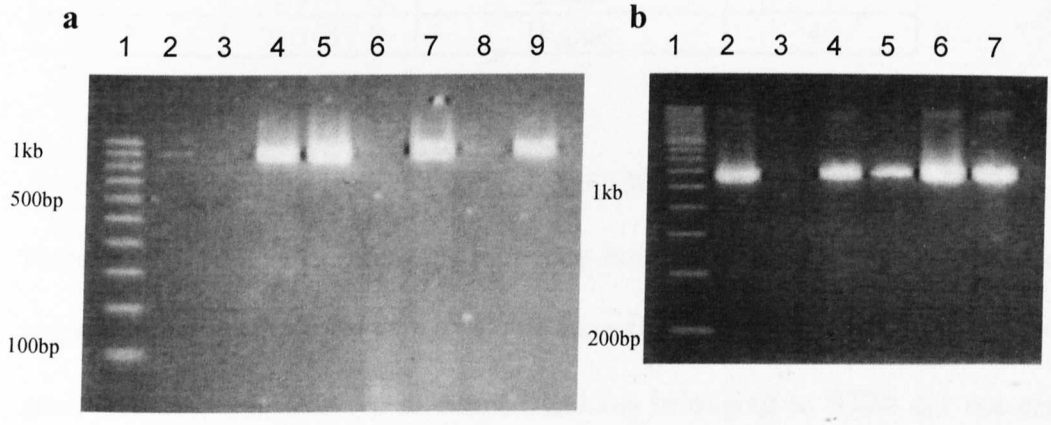



Figure 3.12 a and b Presentation of *cap* 5 and *cap* 8 PCR products. 3.14a Lane 1, 100bp Molecular weight ladder; Lane 2, Reynolds strain positive control; Lane 3 water Control, Lanes 4-9 correspond to isolates in Table 3.13. Figure 3.12b Lane 1, 200bp molecular weight ladder; Lane 2 EMRSA 16 positive control; Lane 3, water control, Lanes 4-7 correspond to isolates in Table 3.14.

Table 3.13 Isolates represented in Figure 3.12a.

| Lane | Isolate | Animal source | ST |
|---|---|---|---|
| 4 | 01.9797.D | Chicken | 5 |
| 5 | 01.7817.X | Human | 15 |
| 6 | 01.9744.S | Horse | 25 |
| 7 | 04.1381.H | Human | 30 |
| 8 | 02.4615.V | Human | 45 |
| 9 | 02.4194.G | Milk | 97 |

Table 3.14 Isolates represented in Figure 3.12b

| Lane | Isolate | Animal source | ST |
|---|---|---|---|
| 4 | 01.1000.N | Human | 5 |
| 5 | 02.4275.G | Cow | 15 |
| 6 | 01.9747.H | Horse | 30 |
| 7 | 05.1017.P | Human | 45 |

Animal isolates belonging to ST5 and ST30 carried the *cap*5 and *cap*8 genes respectively, while the same strains in the human collection carried *cap*8 and *cap*5 respectively. Two STs (ST15 and ST25) from the human isolate collection carried the genes for both capsular types. Animal isolates belonging to ST22 did not carry the genes for *cap*5 or *cap*8. Only isolates belonging to ST45/ST47 carried the same *cap* gene (Table 3.15).

Figure 3.13 Distribution of *cap*5 and *cap*8 genes among animal and human *S. aureus* isolates

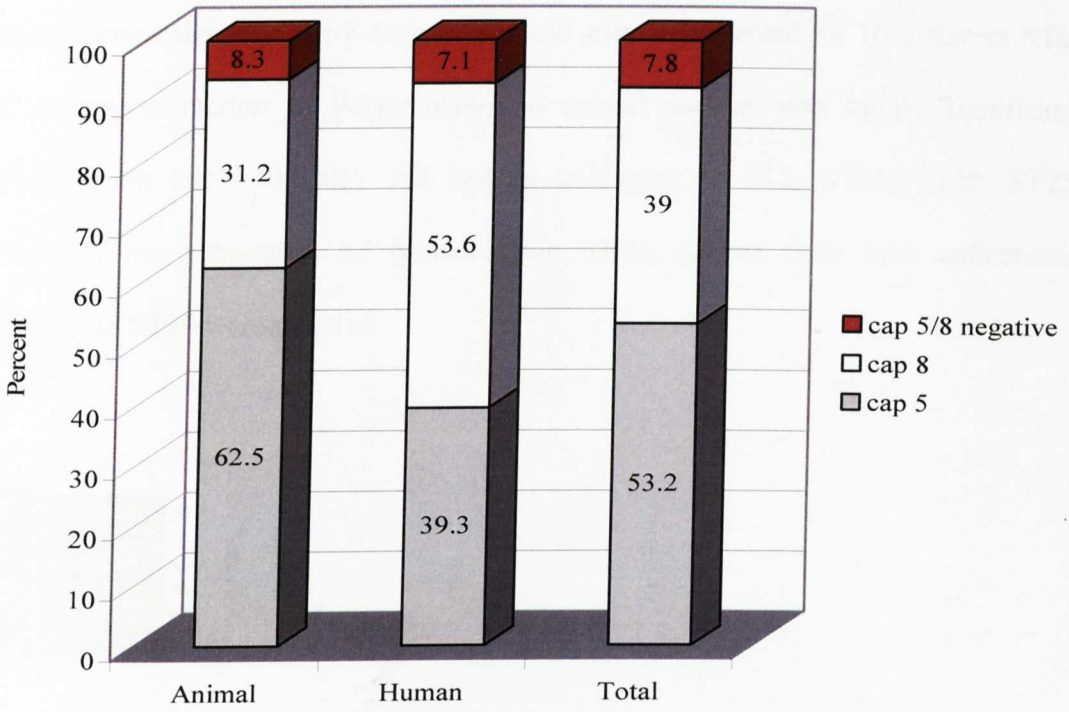



Table 3.15 STs and distribution of *cap*5 and *cap* 8 genes of *S. aureus* from the animal and human isolate collections

| ST | Animal isolates | Human isolates |
|---|---|---|
| ST5 | *cap* 5 | *cap* 8 |
| ST15 | *cap* 8 | *cap* 5 and *cap* 8 |
| ST22 | *cap* 5 and *cap* 8 negative | *cap* 8 |
| ST25 | *cap* 5 | *cap* 5 and *cap* 8 |
| ST30 | *cap* 8 | *cap* 5 |
| ST45/ST47 | *cap* 8 | *cap* 8 |

### 3.3.1.6 Haemolysins α, β, γ, and δ detection

All isolates within the animal and human isolate collections were positive for haemolysins α, γ and δ (Figure 3.14). Twenty nine (37.7%) isolates, 27 animal and 2 human, carried the gene for β-haemolysin and were represented by 10 different STs. The overrepresentation of β-haemolysin in animal isolates was highly significant (Fishers exact test $P$=0.0001). All isolates belonging to ST5, ST15, ST22, ST25 ST45/ST47 were negative for β-haemolysin while isolates from both collections belonging to ST30 were positive.

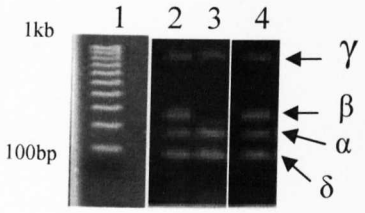



Figure 3.14 Presentation of haemolysin α, β, γ and δ multiplex PCR products. Lane 1. 100bp molecular weight ladder; Lane 2, 04.7486.G positive control strain; Lane 3, 01.9796.B; Lane 4, 02.2799.A

### 3.3.1.7 5′ *coa* RFLP

PCR amplification of the 5′ variable region of the *coa* gene, using primers COAG1 and 4, produced a 1.4kb amplicon in all 77 isolates (Figure 3.15). Subsequent restriction analysis produced 18 RFLP patterns (Figure 3.16). Six RFLP patterns were single isolate. Five RFLP patterns (A, C, E, G and M), containing five or more isolates, were most common, and accounted for 62.3% of isolates.

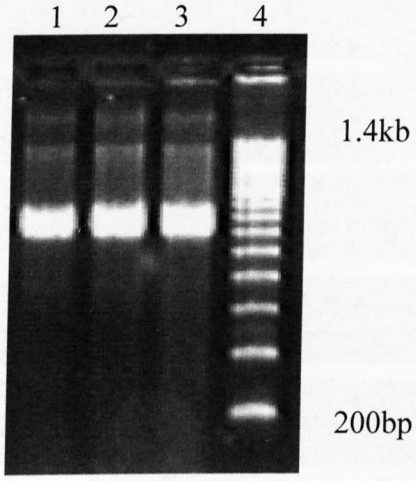



Figure 3.15 5′ *coa* gene PCR products. Lanes 1-3, isolates 01.9768.P, 02.2782.Y and 02.4290.M; Lane 4, 200bp molecular weight ladder

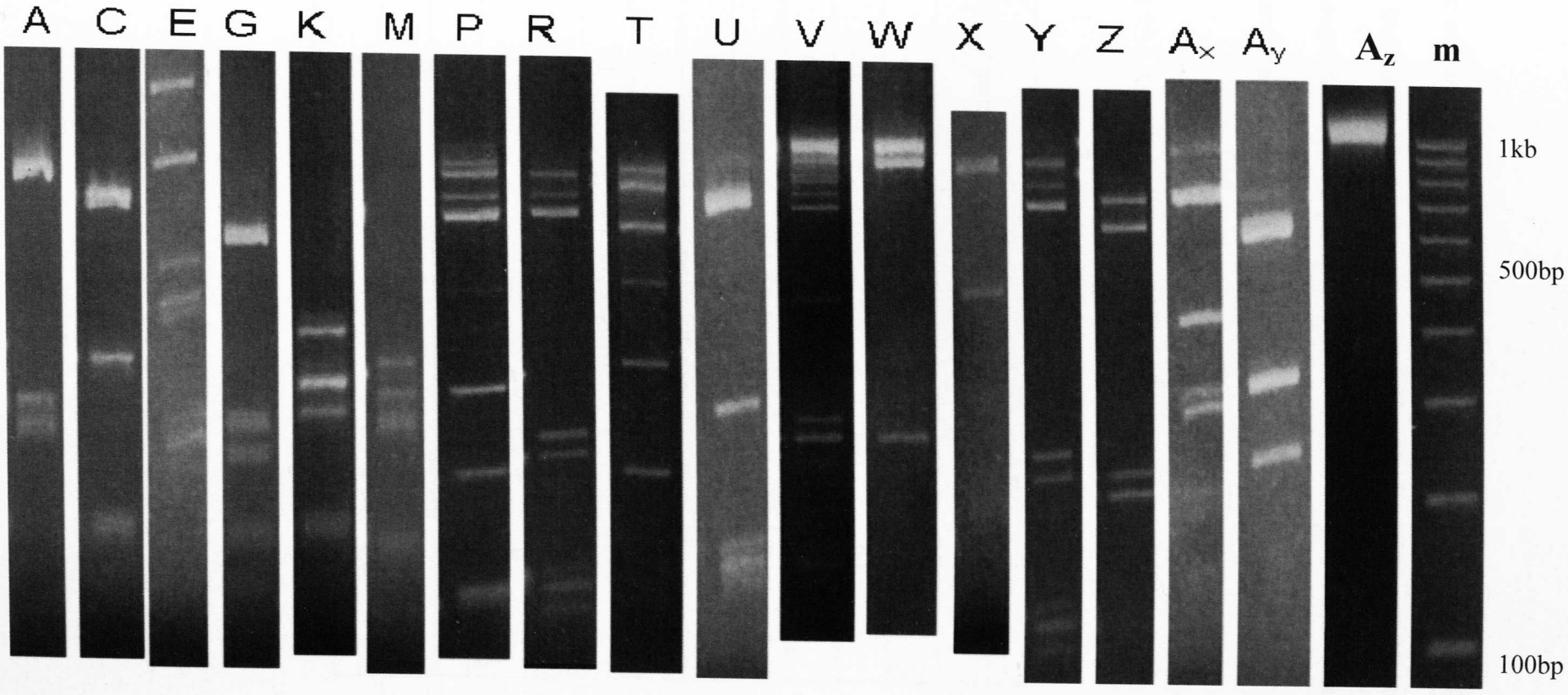



Figure 3.16 Examples of the RFLP patterns of AluI digested 5′ *coa* PCR products. Letters above the lanes indicate the RFLP type; Lane m, 100bp molecular weight ladder

There was a very strong association between ST and RFLP pattern (Table 3.16). Animal and human isolates belonging to ST5, ST15, ST22, ST25 and ST30 shared indistinguishable RFLP patterns. Two STs, ST45 and ST47, produced an amplicon of the correct size but were not digested using AluI (Figure 3.16).

Table 3.16 Association *between ST and 5' coa* RFLP pattern

| RFLP pattern | Associated ST[a] | |
|---|---|---|
| | Animal | Human |
| A | 71 | |
| Az | 47 | 45 |
| C | 9 | |
| E | **25**, 816 | **25** |
| G | **5**, 133 | **5** |
| K | 814 | |
| M | 71, 97, 816 | |
| T | **30** | **30** |
| U | | 8 |
| X | **15** | **15** |
| Y | **22** | **22** |
| Z | | 8 |

[a] STs containing more than one isolate

### 3.3.1.8 3′ coa PCR

Amplification of the 3′ end of the *coa* gene produced eight different amplicons. These amplicons ranged in size from 261bp (3 repeats) to 828bp (10 repeats), reflecting the number of 81bp repeat units contained in this part of the *coa* gene and an 18bp terminal sequence (Figure 3.17).

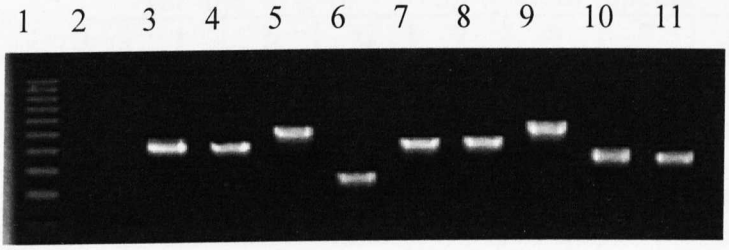



Figure 3.17 Presentation of 3′ *coa* PCR products. Lane 1, 100bp molecular weight marker; Lane 2 NCTC 11047 negative control strain; Lane 3 NCTC 8325 positive control strain; Lanes 4-11, correspond to isolates in Table 3.17

Table 3.17 Isolates represented in Figure 3.17

| Lane | Isolate | Animal source | ST | Number of 81bp tandem repreats |
|---|---|---|---|---|
| 4 | 02.4275.G | Cow | 5 | 5 |
| 5 | 01.9771.Y | Pig | 22 | 6 |
| 6 | 02.4212.M | Milk | 9 | 3 |
| 7 | 01.9702.V | Chicken | 692 | 5 |
| 8 | 01.9762.X | Pheasant | 814 | 5 |
| 9 | 02.4297.H | Cow | 47 | 6 |
| 10 | 01.9796.B | Phillips goose hawk | 5 | 4 |
| 11 | 01.9744.S | Horse | 25 | 4 |

### 3.3.1.9 Combined 5′ and 3′ *coa* data

Combining the *coa* 5′ RFLP typing data with the *coa* 3′ repeat unit data produced 32 coagulase gene types (Table 3.18).

Table 3.18 Coagulase gene types for all animal and human isolates

| *coa* 5′ RFLP | Number of *coa* 3′ 81bp tandem repeats | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| A | | | 1 | 1 | 1 | 3 | | |
| Ax | | 1 | | | | | | |
| Ay | | | | | | 1 | | |
| Az | | | | 4 | | | | |
| C | 9 | | | | | | | |
| E | | 3 | 1 | | 1 | | | |
| G | | 4 | 1 | 7 | 1 | | 2 | |
| K | | | 2 | | | | | |
| M | | | | | | 8 | | 5 |
| P | | | 1 | | | | | |
| R | | | 1 | | | | | |
| T | | 2 | 1 | 1 | | | | |
| U | | | 3 | | | | | |
| V | | | | | 1 | | | |
| W | | | 1 | | | | | |
| X | | 1 | 3 | | | | | |
| Y | | | | 4 | | | | |
| Z | | | 1 | | 1 | | | |
| Total | 9 | 11 | 16 | 17 | 5 | 12 | 2 | 5 |

The combined coagulase typing data grouped ST5, ST15, ST22, ST25, ST30 and ST45/ST47 into 12 coagulase types (Table 3.19). While restriction analysis grouped these STs into distinct groupings, the number of 81bp repeat units was more variable. Only isolates belonging to ST22 and ST45/ST47 formed single coagulase types.

Table 3.19 Comparison of coagulase types among animal and human *S. aureus* isolates belonging to the same ST

| RFLP pattern | Number of 81bp repeat units | | | |
|---|---|---|---|---|
| | 4 | 5 | 6 | 7 |
| Az | | | ST45/ST47[§] | |
| E | ST25[§] | ST25[†] | | |
| G | ST5[§] | ST5[‡] | | ST5[§] |
| T | ST30[§] | ST30[†] | ST30[†] | |
| X | ST15[§] | ST15[†] | | |
| Y | | | ST22[§] | |

[§]Animal and human isolates; [†]Human isolates only; [‡]Animal isolates only

In summary, all isolates were negative for the presence of the genes encoding staphylococcal enterotoxins B and E, epidermolytic toxins A and B and PVL, and positive for heamolysin α, γ and δ. Four virulence factors were over represented within the overall isolate collection. The genes encoding fibronectin binding protein (*fnbA*) and staphylococcal enterotoxin A (*sea*) were overrepresented among the human isolates and the gene encoding β-haemolysin (*hlg-b*) and the production of biofilm was overrepresented among the animal isolates.

The main aim of this study was to examine the presence and distribution of 20 virulence factors in *S. aureus* strains common to both animals and humans. These included MLST sequence types ST5/125, ST15, ST22, ST25, ST30 and ST45/47.

Twelve isolates, seven animal and five human, belonged to ST5 (Table 3.20). All isolates within this group had a predominant *agr* B1C2 amplicon (type A) and exhibited a type G 5′ *coa* RFLP pattern. Animal isolates harboured the gene for capsular polysaccharide type 5. Conversely, the human isolates harboured the gene for capsular polysaccharide type 8. Ten of the 12 isolates had a common B1C2 RFLP pattern and were negative for all toxin genes. Negative results for the presence of *icaA* were only detected among isolates of animal origin.

Isolates belonging to ST15 (3 human and 1 animal) exhibited a predominant *agr* cluster and coagulase type and were all negative for the presence of toxins. The single major difference between animal and human isolates was in the presence of genes encoding capsular polysaccharides (Table 3.20).

Sixteen isolates (7 animal and 9 human) belonged to ST22, ST25, ST30, and ST45. Four isolates (2 animal and 2 human) of ST22 belonged to an identical *agr* cluster and coagulase type. Only human isolates harboured *sec* and *cap*8. There was general agreement of the results for isolates belonging to ST25. All ST25 were positive for *icaA* and biofilm production, negative for β-haemolysin and belonged to identical 5′ *coa* restriction pattern. Only human isolates belonging to ST25 carried the *sea* and *cap*8 genes. Isolates of ST30 belonged to an identical *agr* cluster, and carried the genes encoding SEA and TSST-1. They also produced a common 5′ *coa* restriction pattern, but exhibited variation in the number of 3′ *coa* tandem repeats. All animal isolates harboured the *cap*8 gene only. Isolates belonging to ST45 harboured the *sec* gene and belonged to an identical coagulase type. ST47, a SLV of ST45, contained animal

isolates only and produced a different *agr* B1C2 restriction pattern to those of human origin and harboured the *cap*8 gene only (Table 3.20).

Table 3.20 ST and virulence factor distribution of animal and human *S. aureus*

| CC | ST | Animal/Human | *agr* cluster | Toxins | Coagulase type | *cap* 5 or 8 |
|---|---|---|---|---|---|---|
| 5 | 5/125 | Animal (7) | II-A-2 (6), II-A-3 (1) | *tst* (1) | G-4 (1), G-6 (5), G-7 (1) | 5 |
| | | Human (5) | II-A-2 (2), II-B-2 (1), II-C-1 (1) | Negative | G-4 (2), G-6 (2) | 5 +8 (1), 8 (3) |
| 15 | 15 | Animal (1) | II-A-6 | Negative | X-5 | 8 |
| | | Human (3) | II-A-2 (2), II-B-2 (1) | Negative | X-4 (1), X-5 (2) | 5+8 (3) |
| 22 | 22 | Animal (2) | I-B-1 (1), I-B-8 (1) | Negative | Y-6 (2) | Negative |
| | | Human (2) | I-B-8 (2) | *sec* (2) | Y-6 (2) | 8 |
| 25 | 25 | Animal (2) | I-A-3 (2) | Negative | E-4 (2) | 5 |
| | | Human (2) | I-A-3 (1), I-B-3 | *sea* (1) | E-4 (1), E-5 (1) | 8 |
| 30 | 30 | Animal (1) | III-B-6 | *sea, tst* | T-4 | 8 |
| | | Human (3) | III-A-6 (1), III-B-6 (2) | *sea, tst* (2), *tst* (1) | T-4 (1), T-5 (1), T-6 (1) | 5+8 |
| 45 | 47 | Animal (2) | I-B-4 (2) | *sec* (2) | Az-6 | 8 |
| | 45 | Human (2) | I-B-8 (2) | *sec(2)* | Az-6 | 5 (1), 5+8 (1) |

Numbers of isolates are in parenthesis.

## 3.4 Discussion

### 3.4.1 *agr*

Several authors have reported the existence of distinct restriction patterns within the *agr* groups of *S. aureus*[201]. Polymorphism in the *agr* locus was first described by Ji *et al* and has subsequently been used to characterize *S. aureus* isolates from animals[155]. In this part of the study amplification of the *agr* B1C2 variable region produced four different amplicon types and subsequent restriction analysis of these amplified products identified only eight different restriction types. Gilot and van Leeuwen in an examination of *agr* locus diversification, using AluI RFLP, has reported the presence of only nine restriction types among 322 human and bovine *S. aureus* isolates[155]. Combining *agr* grouping data from chapter two with B1C1 amplicon and restriction pattern data produced 18 *agr* clusters. Two clusters (II-A-2 and I-A-3) accounted for 58.5% of the isolates tested. The distribution of these predominant clusters was relatively even between the animal and human isolate collections. In contrast, Gilot *et al* have reported statistically significant differences in the distribution of *agr* restriction types between *S. aureus* of bovine and human origin[155]. Some restriction types, containing only a few strains, were found to be unique to either the animal or the human strain collections. This has also been reported by Gilot *et al*[156]. Although, they conclude that the variation in distribution of restriction types between bovine and human *S. aureus*, suggests that the two populations are different and indicates that some types probably expand in one or other population due to the possession of unique genetic characteristics[156].

Studies of *S. aureus* from bovine mastitis have limited numbers of *agr* restriction types. The majority of which have belonged to one particular restriction type. It has been concluded that this observation suggest the occurrence of host adapted or tissue adapted *S. aureus* strains in which the *agr* restriction type may play a role[156]. In contrast, animal and human isolates belonging to ST5, ST22, ST25 and ST30 showed identical *agr* clustering while ST15 and ST45/ST47 only differed at the level of B1C2 restriction digest.

### 3.4.2 Toxin profiles

Overall 36.4% of strains carried one or two toxin genes. Taken separately, 26.5% of animal strains and 51.7% of human strains carried one or more toxin gene. These findings are in contrast to data published by Morandi *et al*, where 67% of *S. aureus* isolated from different dairy sources were found to be positive for at least one toxin gene[304]. It has been suggested that SEs carried on MGE are not randomly distributed but are strongly associated with clonal lineages. Four toxin genes were detected among the isolates tested. These were *tst* (11), *sec* (8), *sea* (7) and *sed* (1). Holtfreter *et al* suggest that most SEs are predominantly transferred vertically while horizontal transmission between different lineages is limited[191]. The SE genes from strains in this study were also found in strains of divergent clonal lineages that do not share a common ancestor.

The gene encoding SEA was overrepresented among isolates of human origin and has been shown previously, by Aarestrup *et al* and Kenney *et al*, to be the predominant enterotoxin type among human *S. aureus*[6,224]. *sea* and *tst* have been shown to be significantly correlated to ST30, and were detected together among the animal and

human *S. aureus* isolates belonging to ST30 but not in any other clonal background. The gene encoding TSST-1 has been shown to be carried by a family of related *S. aureus* pathogenicity islands (SaPI). It has been reported that these SaPIs do not spread between different genetic backgrounds or the efficiency of transfer is low[302]. Waldron *et al* suggest that barriers to horizontal transfer could be due to the incompatibility of related bacteriophages, SaPIs and plasmids; varying susceptibility to transduction or conjugation; or the presence of the SauI restriction modification system[463]. Only a single animal isolate of ST5 and animal and human isolates of ST30 carried *tst* suggesting that horizontal transfer of *tst* may be an infrequent event. Variations within the toxic shock syndrome toxin gene have been shown by Ho *et al*[189] and Lee *et al*[254]. They were able to show that a TSST protein from *S. aureus* isolated from a sheep showed complete identity with classical TSST-1 but exhibited a different isoelectric point and was therefore termed TSST-$1_{ovine}$[254]. No clonal background was given for these isolates therefore the significance of this finding to animal isolates carrying *tst*, in this study, is unclear.

Various studies have reported the presence of *sec* among animal *S. aureus* strains, particularly those of bovine origin[16,146]. The *sec* gene was the second most prevalent virulence gene detected. While there was no overrepresentation of the *sec* gene, between the animal and human isolate collections, only isolates belonging to ST22 and ST45/47 carried *sec*. The remaining common STs were all *sec* negative. As has been shown with *tst*, the *sec* gene has been shown to be heterogeneous containing several antigenic and sequence molecular variants, designated SEC1, SEC2, SEC3, $SEC_{bovine}$, $SEC_{canine}$ and $SEC_{ovine}$ [282,298,372]. These variants have been classified on the basis of minor antigenic differences and the animal host with which they are associated. Thus, it

may not just be the presence or overrepresentation of a particular toxin gene that determines possible host specificity, but the molecular structure or biochemical properties of certain genes or proteins may determine host or tissue adaptation of *S. aureus* strains, a concept also suggested by Zadoks *et al*[497].

Among animal and human *S. aureus* strain collections, with a common clonal background, there was a low rate of variation in virulence gene content. Diep *et al* have also shown that there was an infrequent change in virulence gene detection in closely associated *S. aureus* genotypes from humans[105]. These data are in contrast to that of van Leeuwen *et al* who have shown significant variations in virulence gene content between *S. aureus* strains from cases of bovine mastitis and humans[452].

All isolates were negative for the genes encoding SEB, SED, SEE, ETA, ETB and PVL. In a study by Stephen *et al* none of the genes encoding SEB, SEE, ETA or ETB were found in *S. aureus* isolated from cattle[410]. In contrast, human *S. aureus* strains have been shown to carry the epidermolytic toxin genes, *eta* and *etb*, at a low frequency[33]. This has also been shown to be the case for *S. aureus* isolated from a variety of animal species[117,454].

While the genes encoding PVL were not detected in either animal or human *S. aureus* isolates, in the present study, high levels of PVL positive community acquired MRSA (CA-MRSA) have been reported in the USA caused by strain USA300 (MLST ST8) and by community acquired MSSA[37]. Ellington *et al* suggest that this higher prevalence has occurred against a background of increasing CA-MRSA rates within the USA which has not been mirrored across Europe[115]. Clinically, PVL has been linked to

necrotizing skin lesions such as furuncles and carbuncles and to a severe form of necrotizing pneumonia especially in previously healthy young adults[44]. Rankin *et al* have reported the presence of PVL-positive MRSA among companion animals that produced identical infections to those found in humans and make two possible conclusions; companion animals infected by PVL-positive MRSA strains may act as a reservoir of infection for humans, or these strains are human in origin and companion animal are infected by their owners[346].

### 3.4.3 Biofilm production

Seventy three (94.8%) isolates were positive for *icaA*, 79.2% of isolates positive for biofilm production, and 75.3% were positive for both *icaA* and biofilm. The production of biofilm was significantly overrepresented among strains of animal origin. The high prevalence of biofilm producing *S. aureus* has also been reported from isolates causing bovine mastitis. Over 50% of the animal isolates tested were of bovine origin and may account for the overrepresentation. Animal and human strains belonging to ST5, ST22 and ST25 were positive for *icaA* and biofilm production as were animal isolates of ST30 and ST47. Three animal isolates were *icaA* PCR negative yet were still able to produce a biofilm. Evidence is now emerging for the existence of an *icaA*-independent biofilm mechanism in *S. aureus*. The findings of O'Gara indicate that other surface proteins, which typically function as antigenic determinants or in binding to extracellular matrix proteins, may also act as biofilm adhesins[317]. A report by Crampton *et al* has shown that differences in biofilm production between the two strain populations could be a result of the growth conditions used, lack of some biofilm to adhere to the polystyrene microtitre plates due to possible differences in adherence mechanisms or the presence of imperfections in the microtitre plate[323]. The biofilm

assays were repeated in triplicate with all strains cultured on the same plate suggesting a real difference between the two strain collections.

### 3.4.4 *fnbA*

*Staphylococcus aureus* fibronectin-binding proteins A and B (FnBPs) are encoded by one of two *fnb* genes (*fnbA* and *fnbB*)[330]. These proteins play an important role in *S. aureus* pathogenesis by promoting bacterial attachment to intravenous catheters and orthopedic prosthesis[167]. Although *S. aureus* is considered an extracellular pathogen, it has been well documented that the FnBPs also mediate bacterial invasion of cells[379]. Fifty (64.9%) of the isolates tested were positive for *fnbA*. Of the STs common to both isolate collections those belonging to ST15, ST22 and ST45/ST47 were positive for *fnbA*. A study by van Leeuwen *et al* investigating host and tissue-specific traits in *S. aureus* reported that the genes encoding adhesion proteins, including *fnbA*, were evenly distributed among human and veterinary strains[452]. In contrast, the presence of *fnbA* among isolates in this study was significantly overrepresented among human *S. aureus* strains. A low frequency of *fnbA* negative *S. aureus* isolates from humans, which were able to bind fibronectin, has been reported by Peacock *et al*[330]. These isolates carried the *fnbB* gene. Therefore, it is possible that the *fnbA* negative animal isolates in this study may harbour the *fnbB* gene.

### 3.4.5 Capsule typing

Capsular polysaccharides, most commonly those of serotype 5 or 8, are produced by most human *S. aureus* isolates independently of their source[407]. In a survey of clinical *S. aureus*, from human patients in a trauma unit, Na'was was able to show that 87.7% of isolates possessed a capsular polysaccharide, type 5 capsule (43.1%) or type 8 capsule

(44.6%)[308]. Studies on the prevalence of encapsulated strains in cattle, sheep and goats have shown a different situation, with variable prevalence of strains producing either capsular type 5 or 8 in different animal species and different countries. Poutrel *et al* has shown that 73% of bovine, ovine and caprine *S. aureus* produced polysaccharide type 5 or 8 capsule[340]. These isolates showed a distinct difference in distribution of these capsular types among these animals. Among bovine isolates type 5 was predominant (51.4%) while type 8 was found in less than 20% of isolates. Conversely, capsular type 8 was significantly more frequent in caprine and ovine strains, 68.5% and 75.8%, respectively, with type 5 significantly less frequently detected. In the present study 92.2% of strains were positive for *cap*5 or *cap*8. Six strains were negative for both and included isolates of ST22 from animals. Of the STs common to both isolate collections only human isolates belonging to ST45/ST47 carried identical capsular polysaccharide genes. Among strains from this study the *cap*5 gene was significantly overrepresented among animal strains. While there was a difference in the overall distribution of the *cap*8 gene between animal and human strains the difference was not statistically significant. An investigation of capsule expression by bovine *S. aureus* performed by Sordelli *et al*, reported an equal but low frequency distribution of *cap*5 and *cap*8. Surprisingly, 86.3% of isolates in that study were negative for both capsular types compared to the 7.8% in this study[407]. Sordelli *et al*, suggest that their strains, like those of Poutral *et al*, may carry an intact copy of the *cap*5 gene cluster that may have a mutation in the *cap5E* rendering them capsule negative[252].

### 3.4.6 Haemolysin typing

All isolates in this study harboured the genes for α, γ and δ haemolysins. The phenotypic detection of α, β, δ haemolysins and genotypic detection of γ-haemolysin by Peacock *et al*, among *S. aureus* from human carriers and those with invasive disease, has also shown a high frequency of α, γ and δ haemolysins (range 69%-100%)[332]. In contrast, Aarestrup *et al* has shown that the phenotypic expression of α-haemolysin in *S. aureus*, from bovine and human sources, to be considerably lower[6]. γ-haemolysin has been shown to be produced by virtually all *S. aureus* strains and is coexpressed along with α-haemolysin[294]. While β-haemolysin can be produced by a large number of *S. aureus* strains, it is particularly prevalent among isolates of bovine origin[107]. This was the case in this study where 78% of the animal isolates carrying the β-haemolysin gene were of bovine origin. Only one isolate within the human isolate collection (ST30) carried the β-haemolysin gene as did the ST30 from the animal isolate collection.

### 3.4.7 Coagulase typing

PCR amplification of the 5′ variable region of the *coa* gene produced an amplicon of the expected size in all 77 isolates. Subsequent endonuclease digestion of this amplicon produced 18 restriction types. A study by Carter *et al* showed a similar number of coagulase types at the 5′ end of the gene among human and animal *S. aureus* isolates from the UK, Italy, Russia and Malaysia[63]. In this study five RFLP patterns (A, C, E, G and M) accounted for 65.8% of isolates. Similarly, discrete populations of *S. aureus* were also identified in a study by Carter *et al*, although a much higher proportion (93%) of the MRSA isolates examined were assigned to one of only five major 5′ *coa* types[63]. Eleven of 14 STs, containing more than one isolate represented in this study, including ST5, ST15, ST22, ST25 and ST30 produced unique restriction patterns. It has been

suggested that coagulase evolution and the evolution of the clonal background, as defined by the ST, are intimately related[63]. This intimate association is consistent with the 5′ *coa* data from this study. In contrast, the novel ST816 produced two different restriction patterns that appear to be more closely associated to animal source than ST.

Amplification of the 3′ (C-terminal) end of the *coa* gene produced eight different amplicons. The number of repeating units ranged from three (261bp) to ten (828bp). Numerous authors have shown variable numbers of repeating units among *S. aureus* strains from humans, goats and cattle[169,213-215,218,370,398]. Amplification of the 3′ end of the *coa* gene has been shown to be an unsuitable single method for the characterization of *S. aureus*. Investigation of a number of recognized control strains has variations in the number of tandem repeating units[213,214]. These variations are thought to be caused by either deletion or insertion of repeat units[386]. Also, RFLP of the amplified products of the *coa* gene C-terminal has shown that unrelated strains could be linked to outbreak clusters, thus limiting the effectiveness of this technique for typing unrelated strains[386]. In this study animal and human isolates belonging to ST5, ST15, ST22, ST30 and ST45/ST47 were placed into distinct restriction groupings that produced variable numbers of 3′ tandem repeats. Therefore, PCR amplification of the 3′ *coa* gene, was not a useful comparative technique.

There is a general consensus that *S. aureus* from animals and humans constitute separate populations consistent with the concept of host specificity[401,401,497]. The aim of this part of the study was to examine a range of virulence factor from *S. aureus* from animals and humans, many of which were genotypically closely related, and to compare these data to determine clonal similarities. The data show that isolates of a given clonal

background share common genetic characteristics. Overrepresentation of the virulence genes encoding β-haemolysin, *fnbA*, *sea*, and *cap5* and biofilm production between animal and human *S. aureus* strains suggest that there may be a degree of host adaptation. Isolates of ST5, ST15, ST22, ST25, ST30 and ST45/47 representing five of the major human *S. aureus* clonal backgrounds, and detected in both animal and human strains, shared virulence factors indicating an evolutionary relationship that would suggest horizontal transfer of these clones between animals and humans.

## Chapter 4 Antimicrobial susceptibilities

### 4.1 Introduction

Increasing antimicrobial resistance has become a serious concern worldwide. Over the past 35 years there has been an intense debate over the impact of the use of antimicrobial agents in food animals and their effect on human health[345]. To date there has been no definitive answer to this question. Therefore, the agricultural use of antibiotics remains controversial because of the possibility of drug resistant strains being transferred to humans via contaminated food[445]. Large quantities of antibacterial substances are used in the production of food and in companion animals[261,342]. These include antibiotics for treatment, prophylaxis and until recently growth promotion. Biocides are used to reduce or eliminate environmental contamination, preserve foods and in the case of copper sulphate also as a mineral supplement in animals[4]. Aarestrup *et al* have reported that ten times more biocides are used in the production of food than antibiotics[4]. Recently concerns have arisen regarding the potential emergence of cross-resistance and co-resistance between widely used disinfectants and antibiotics[285]. There are few reports on the antimicrobial susceptibility of *S. aureus* isolates from animals in the UK[5]. This present study was conducted to determine the frequency of antibiotic and biocide resistance among *S. aureus* isolates from a range of animal species, to agents commonly used in human clinical practice and in the production of food animals and to compare the regional variations in antimicrobial resistance across the four geographic locations.

## 4.2 Materials and methods

### 4.2.1 Staphylococcal isolates

A total of 233 *S. aureus* were examined for antimicrobial resistance (Table 2.1 and Figure 4.1)


Figure 4.1 Geographic sources of 233 animal *S. aureus* isolates.

### 4.2.2 Disc diffusion testing

Disc diffusion susceptibility testing was carried out, using the antimicrobials listed in Table 4.1, following the recommendations of The British Society for Antimicrobial Chemotherapy (BSAC)[20]. Four morphologically similar colonies were touched with a sterile loop, and transferred into 2ml of sterile distilled water (Biomerieux, France). The organism suspension was adjusted to McFarland 0.5 turbidity standard (1- 2 x $10^8$ colony forming units (cfu/ml)) using sterile distilled water and a working solution produced by performing a 1:10 dilution (1 x $10^7$ cfu/ml). Isosensitest agar plates (ISA) (Oxoid, UK) were inoculated, allowed to dry prior to the application of antibiotic discs and incubated at 35-37°C in air for 18-20 hours. For oxacillin susceptibility testing ISA supplemented with 2% NaCl was used and incubated at 30°C in air for 24 hours. After incubation the zones of inhibition were measured and susceptibility interpretation made based on BSAC criteria.

Table 4.1 List of antimicrobial agents

| Antimicrobial agent | Disc code | Concentration (μg) | Suppliers |
|---|---|---|---|
| Amikacin | AK | 30 | Oxoid, UK |
| Chloramphenicol | C | 10 | |
| Ciprofloxacin | Cip | 1 | |
| Clindamycin | Da | 2 | |
| Erythromycin | E | 5 | |
| Fusidic acid | Fa | 10 | |
| Gentamicin | Cn | 10 | |
| Kanamycin | K | 30 | |
| Linezolid | LZD | 10 | |
| Mupirocin | Mup | 5 | |
| Oxacillin | Ox | 1 | |
| Penicillin | P | 1 unit | |
| Quinupristin/Dalfopristin | QD | 15 | |
| Rifampicin | Rd | 2 | |
| Streptomycin | S | 10 | |
| Sulphamethoxazole | Rl | 25 | |
| Teicoplanin | Tec | 30 | |
| Tobramycin | Tob | 5 | |
| Trimethoprim | W | 5 | |
| Tylosin | Ty | 30 | Mast, UK |
| Vancomycin | Va | 5 | Oxoid, UK |

### 4.2.3 Biocide Minimum Inhibitory Concentration (MIC) agar dilution assay

Isolates were tested for biocide MIC using the agar dilution method for measuring the MIC of various biocides based on the NCCLS methodology (approved standard, January 1997, M7-A4; vol 17, No2). Muller Hinton II cationic adjusted agar plates (Becton Dickenson, UK), using 20ml of agar, were prepared following the manufacturers instructions, and contained biocides in the ranges seen in Table 4.2. Each MIC had growth control plate, containing no biocide, inoculated at the beginning and end of each test batch. Growth control plates for triclosan MIC also included ethanol control plates containing 640µl, 160µl, 80µl and 10µl of 50% ethanol.

The biocide control isolates used were *Staphylococcus aureus* ATCC 29213, NCTC12201, and NCTC 1132.

Table 4.2 List of microbicides

| Biocide | Supplier | Solvent | MIC range |
|---|---|---|---|
| $H_2O_2$ | Sigma-Aldrich | Sterile distilled water | 0.001-0.064% |
| Triclosan | | 50% Ethanol | 0.015-32µg/ml |
| Benzakonium chloride | | Sterile distilled water | 0.5-256µg/ml |
| Cetylpiridinium chloride | | | 0.5-256µg/ml |
| Formaldehyde | | | 0.008-0.1% |
| Chlorhexadine | | | 0.25-64µg/ml |
| Copper sulphate | | | 2-12mM |

Inocula were prepared as above (section 4.2.2)

The working strength inocula were vortexed and 1 ml added to the appropriate well of the multipoint inoculator replicator block (Denlay, UK). Each test plate was marked to indicate the orientation of inoculation. Starting with a negative control, then followed by the lowest biocide concentration and working up the range and ending with a final negative control, each plate was inoculated with 1-2µl of test organism (cell concentration $10^4$ cfu/spot) using a Denlay multipoint inoculator (Denlay, UK). Each plate was left at room temperature until each spot had dried then incubated at 37°C for 16-24 hours. The MIC was defined as the lowest concentration of antimicrobial agent that completely inhibited growth.

## 4.3 Results

### 4.3.1 Antibiotic susceptibilities of animal *S. aureus* isolates from Scotland and Northern Ireland

A total of 233 *S. aureus* were examined for antibiotic susceptibility from livestock, wild and companion animals. Thirty isolates (12.9%) were fully susceptible to the 22 antibiotics tested. Of those isolates resistant to one or more antibiotics, 84.2% were resistant to one or two antibiotics and 15.8 % were resistant to more than three and were considered as multiresistant (Figure 4.2)[316].

Penicillin resistance was detected in 153/233 (65.2%) isolates and was the most frequent type of resistance observed followed by tetracycline (31.8%), sulphamethoxazole (20.2%), ciprofloxacin (19.7%) and streptomycin (10.7%) (Figure 4.3). Fifty one (21.9%) isolates exhibited resistance to antibiotics other than penicillin. No resistance to fusidic acid, gentamicin, kanamycin, linezolid, mupirocin, oxacillin, quinupristin/dalfopristin, teicoplanin or vancomycin was detected using the BSAC testing criteria.

Figure 4.2 Frequency of antibiotic resistant *S. aureus* isolates from animals in Scotland and Northern Ireland

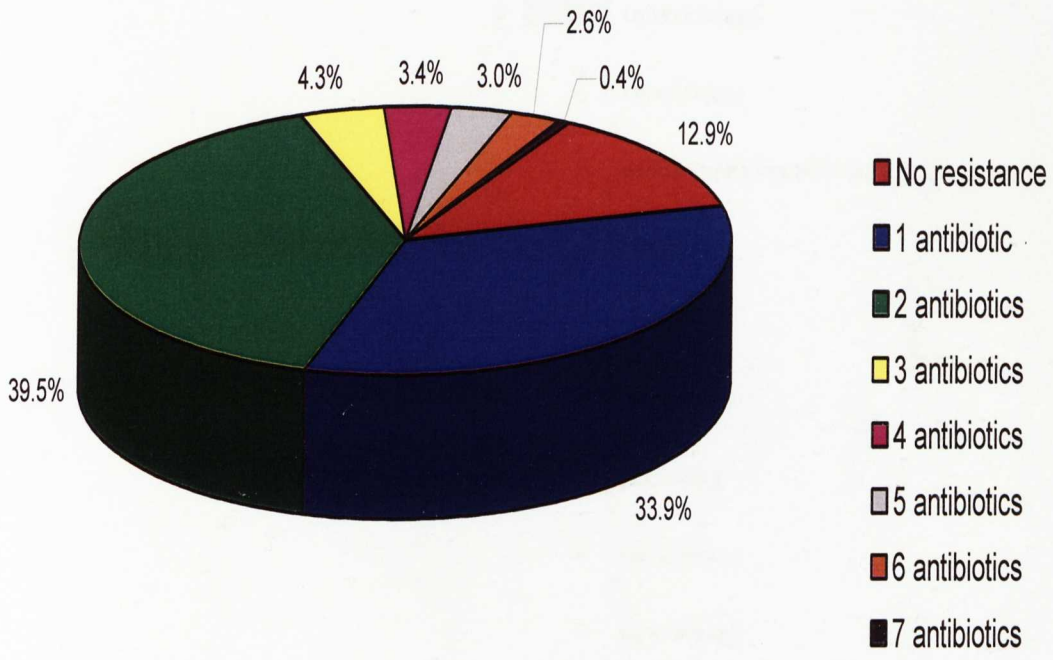
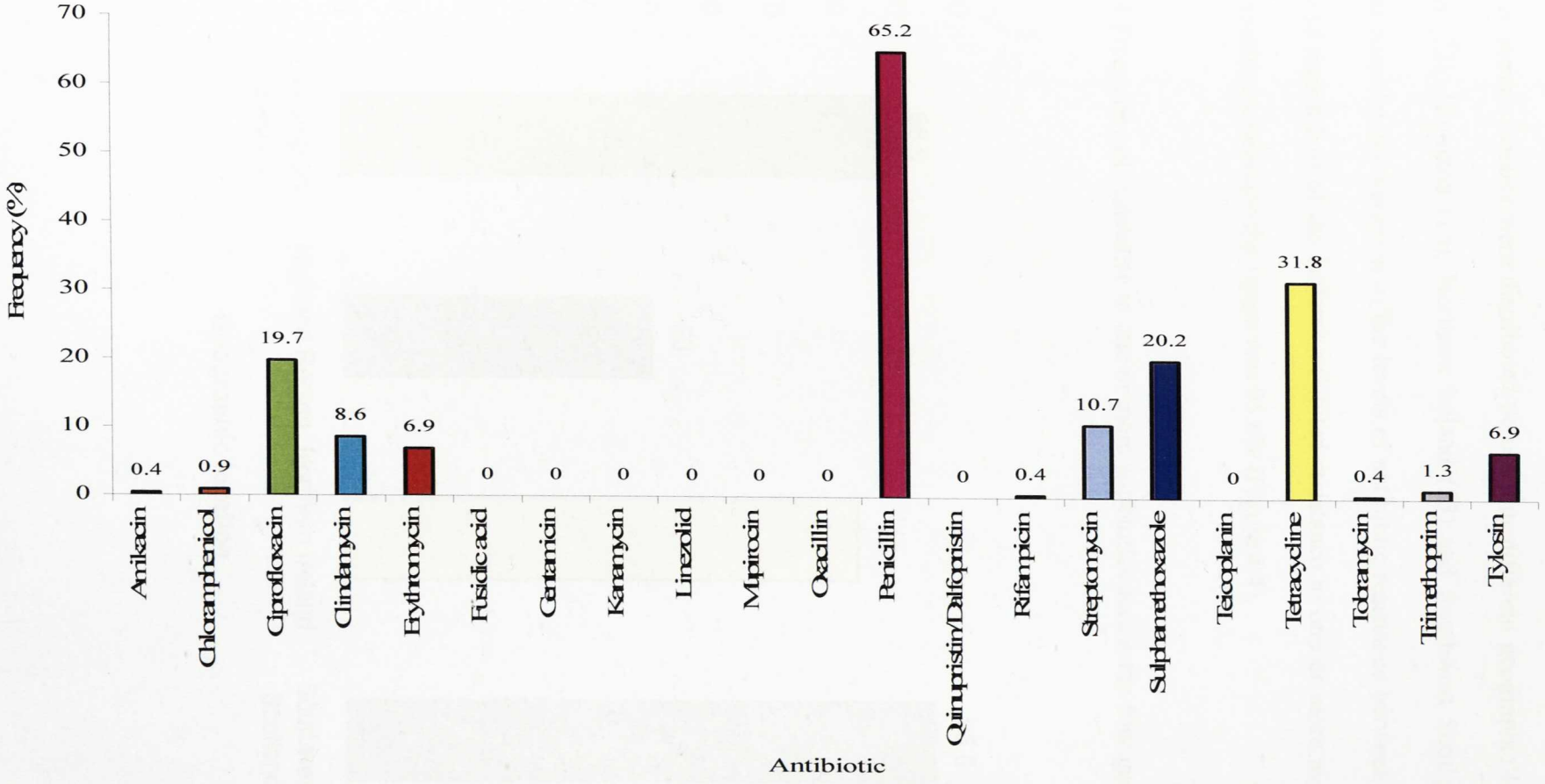
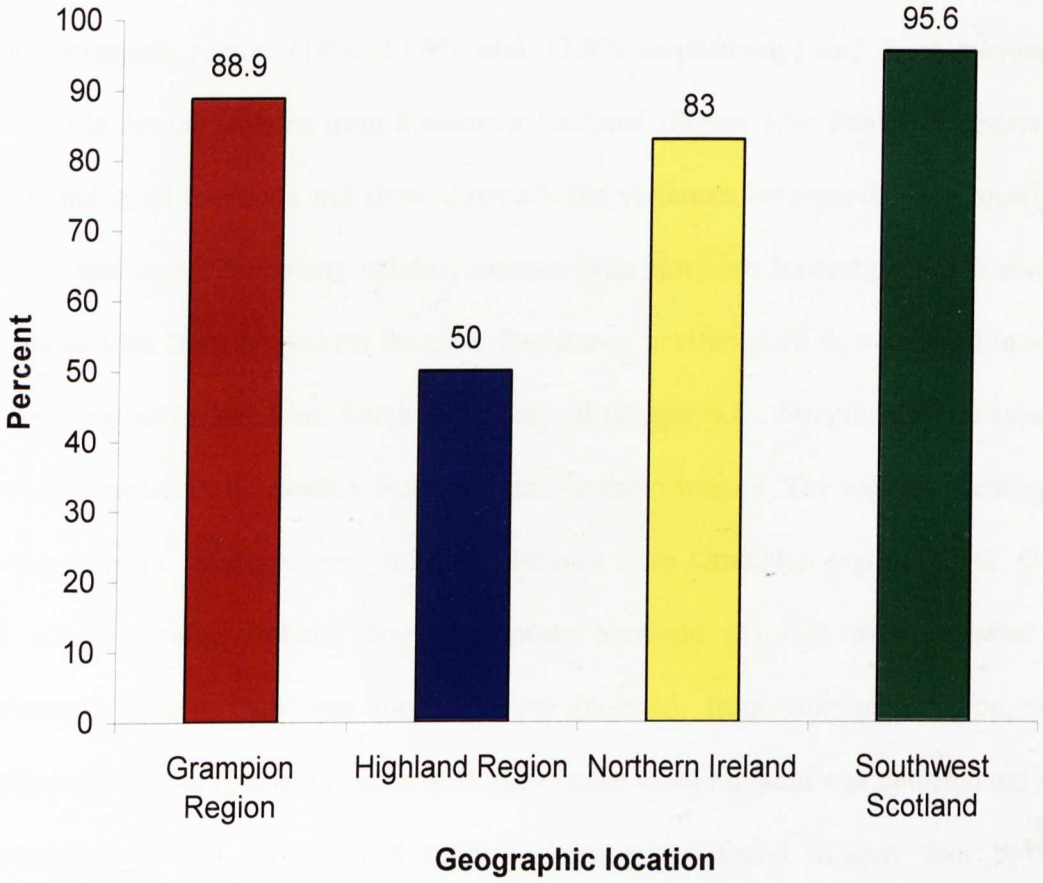


Figure 4.3 Frequency of resistance in *S. aureus* in animals in Scotland and Northern Ireland



The 233 *S. aureus* isolates were distributed across four different geographic locations: Grampian (72), Highland (18), Northern Ireland (53) and Southwest Scotland (90). There was considerable variation in the levels of antibiotic resistance between isolates. In Highland region half of the isolates exhibited resistance to one or more antibiotics, while in Southwest Scotland the figure was 95.6% (Figure 4.4).

Figure 4.4 Frequency of resistance to one or more antibiotics across the four geographic locations.



The frequency of antibiotic resistance of animal *S. aureus* isolates across the four geographic locations is seen in Figures 4.5, 4.6, 4.7 and 4.8. Ciprofloxacin, clindamycin, penicillin and tetracycline resistance were the only resistances detected in all four locations. Resistance to Amikacin was only found in an isolate from a pheasant from Highland region and chloramphenicol resistance only in a bovine isolate from Grampian region. The highest occurrence of ciprofloxacin resistance was found in chicken and a Phillips goose hawk isolate from Northern Ireland (45.3%) and in bovine isolates from Southwest Scotland (18.9%). Resistance to clindamycin and the macrolides, erythromycin and tylosin was most frequently detected in bovine isolates from Grampian region (18%, 13.9% and 13.9% respectively) and least frequently detected in bovine isolates from Southwest Scotland (Figure 4.8). Penicillin resistance was found in all locations and showed remarkable variances between the four locations from as low as 18.9% among chicken isolates from Northern Ireland to 91.1% among bovine isolates from Southwest Scottish. Resistance to rifampicin was detected in only a single equine isolate from Southwest Scotland (Figure 4.8). Streptomycin resistance was not detected in the poultry isolates from Northern Ireland. The highest occurrence of streptomycin resistance was in bovine isolates from Grampian region (25%). Over half of the bovine isolates from Southwest Scotland (51.1%) were resistant to sulphamethoxazole. Only one further isolate (bovine), from Grampian region, was sulphamethoxazole resistant (Figure 4.5). Resistance to tobramycin was detected only in Grampian region (1.4%). Trimethoprim resistance was found in only four bovine isolates, two from Grampian and two Highland (Figure 4.5 and 4.6 ).

Figure **4.5** Frequency of antibiotic resistance in animal *S. aureus* isolates in Grampian region

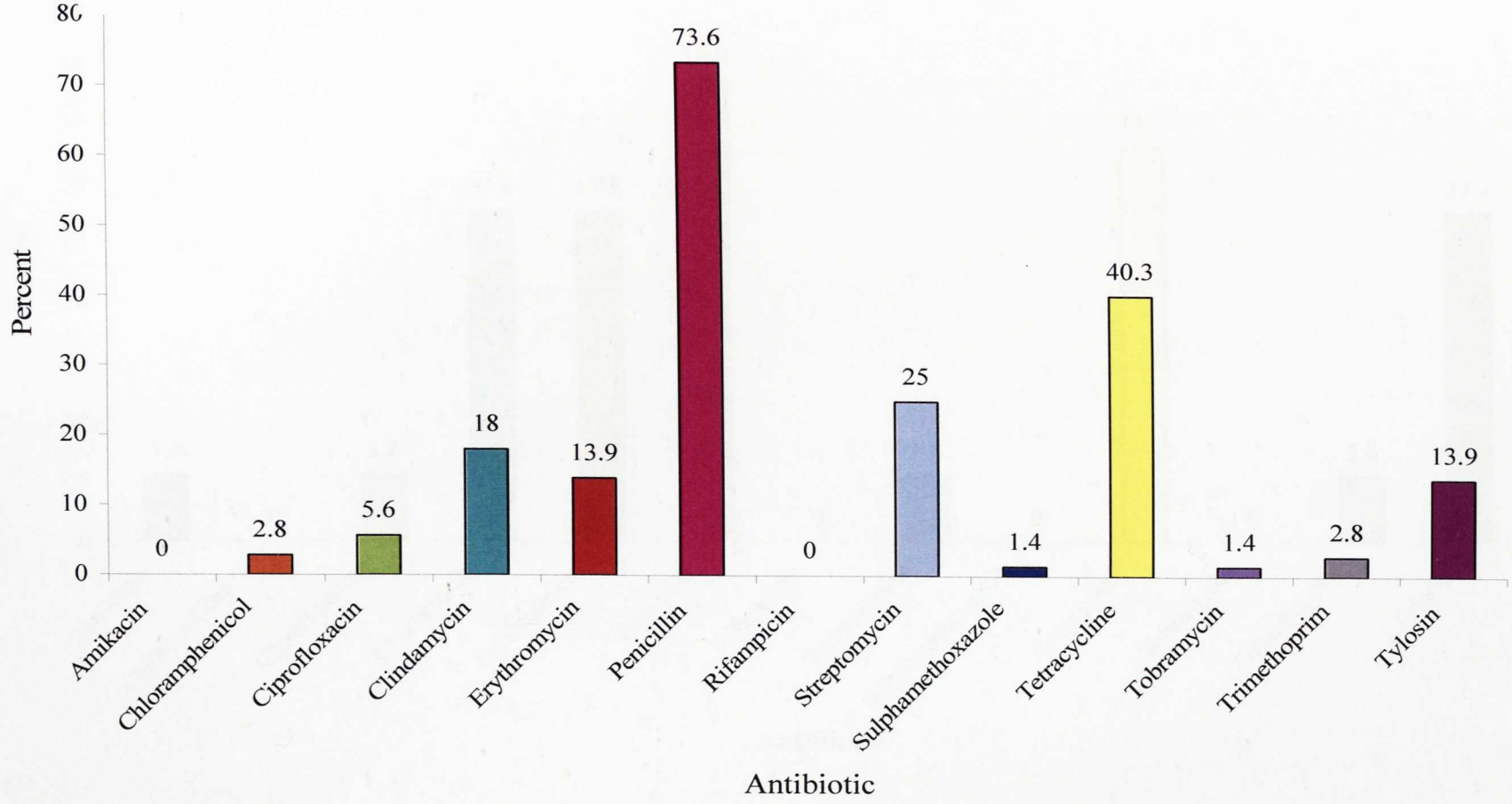


Figure 4.6 Frequency of antibiotic resistance in animal *S. aureus* isolates in Highland region

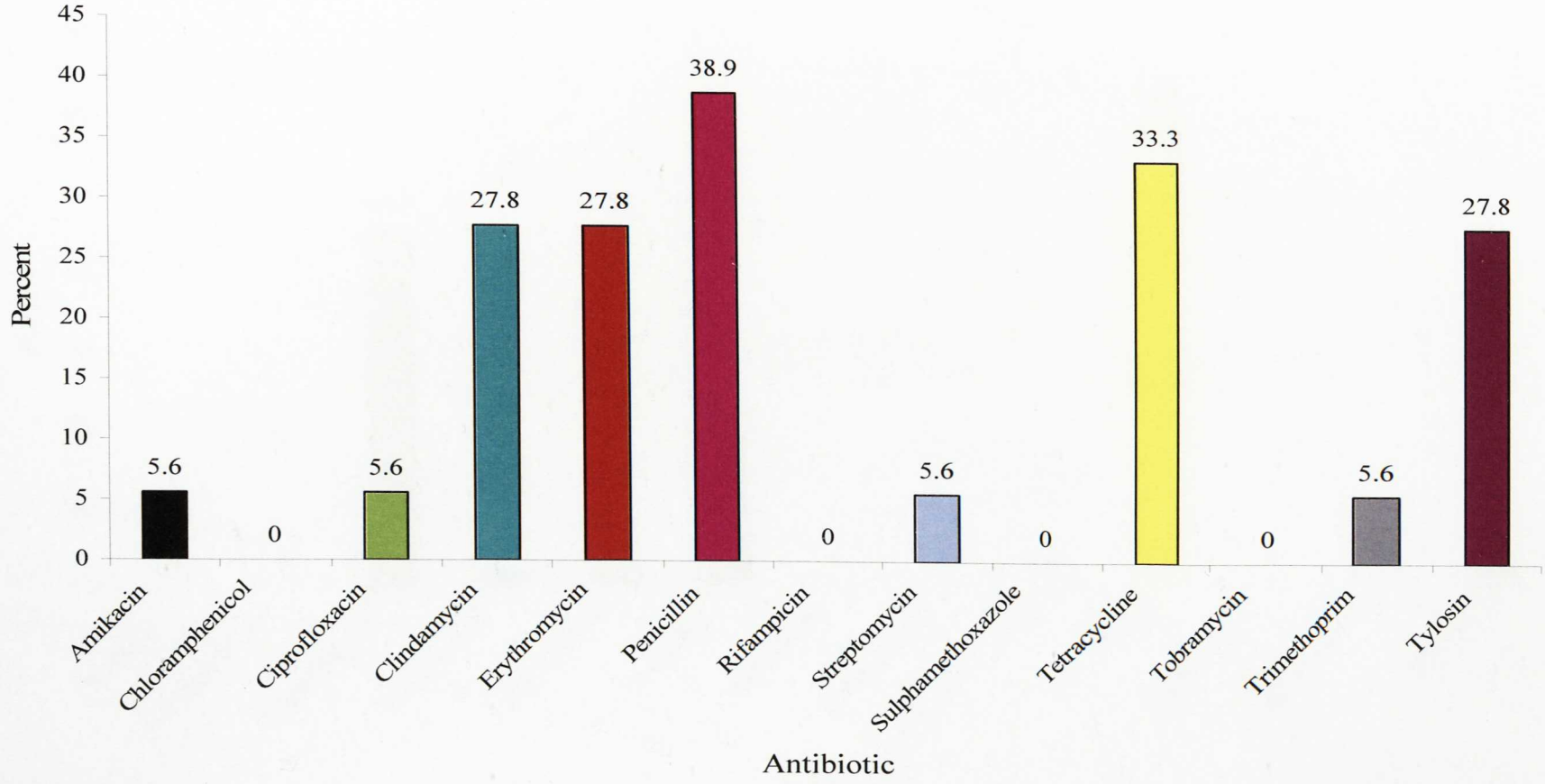


Figure 4.7 Frequency of antibiotic resistance in animal *S. aureus* isolates in Northern Ireland

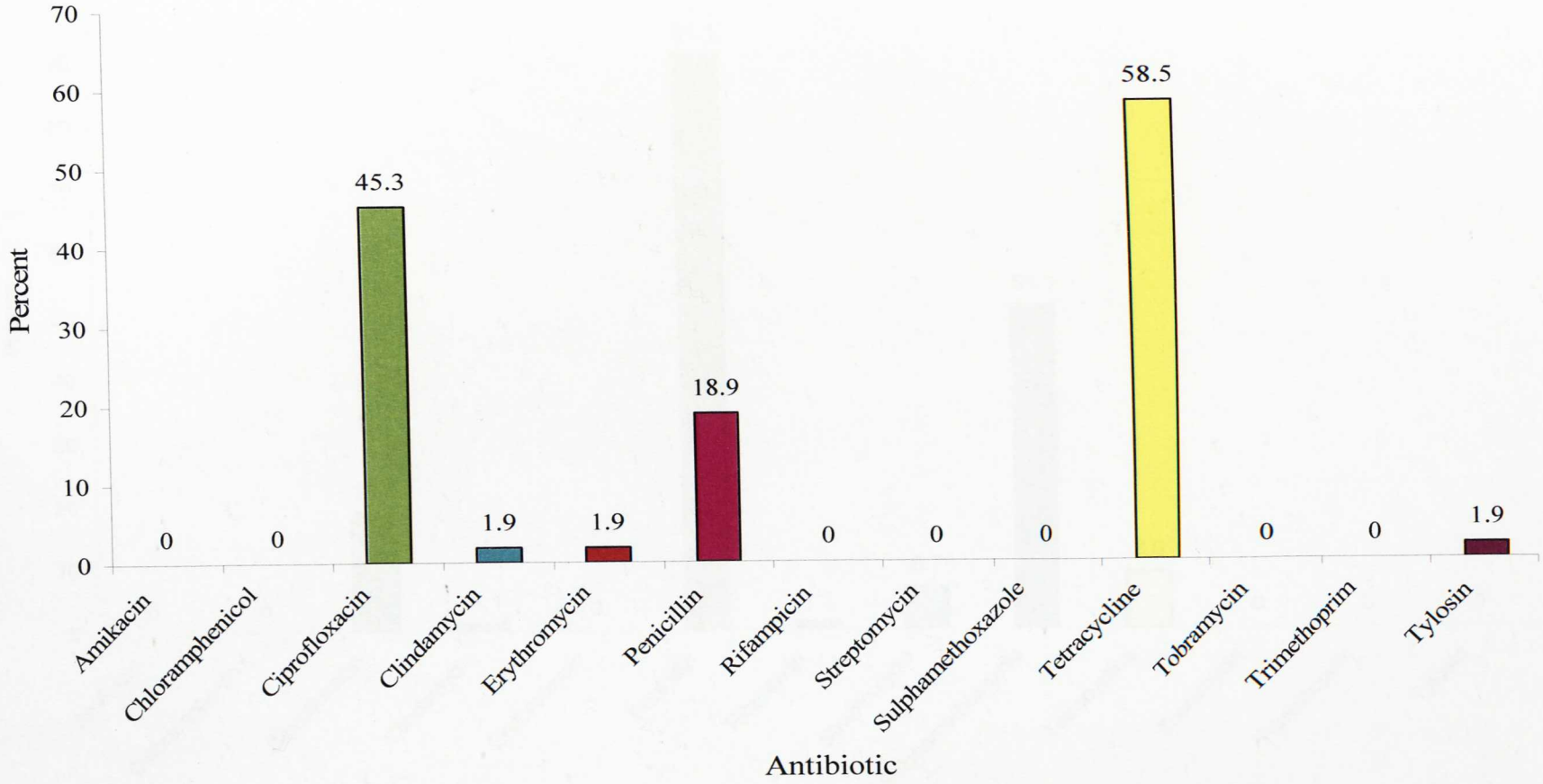
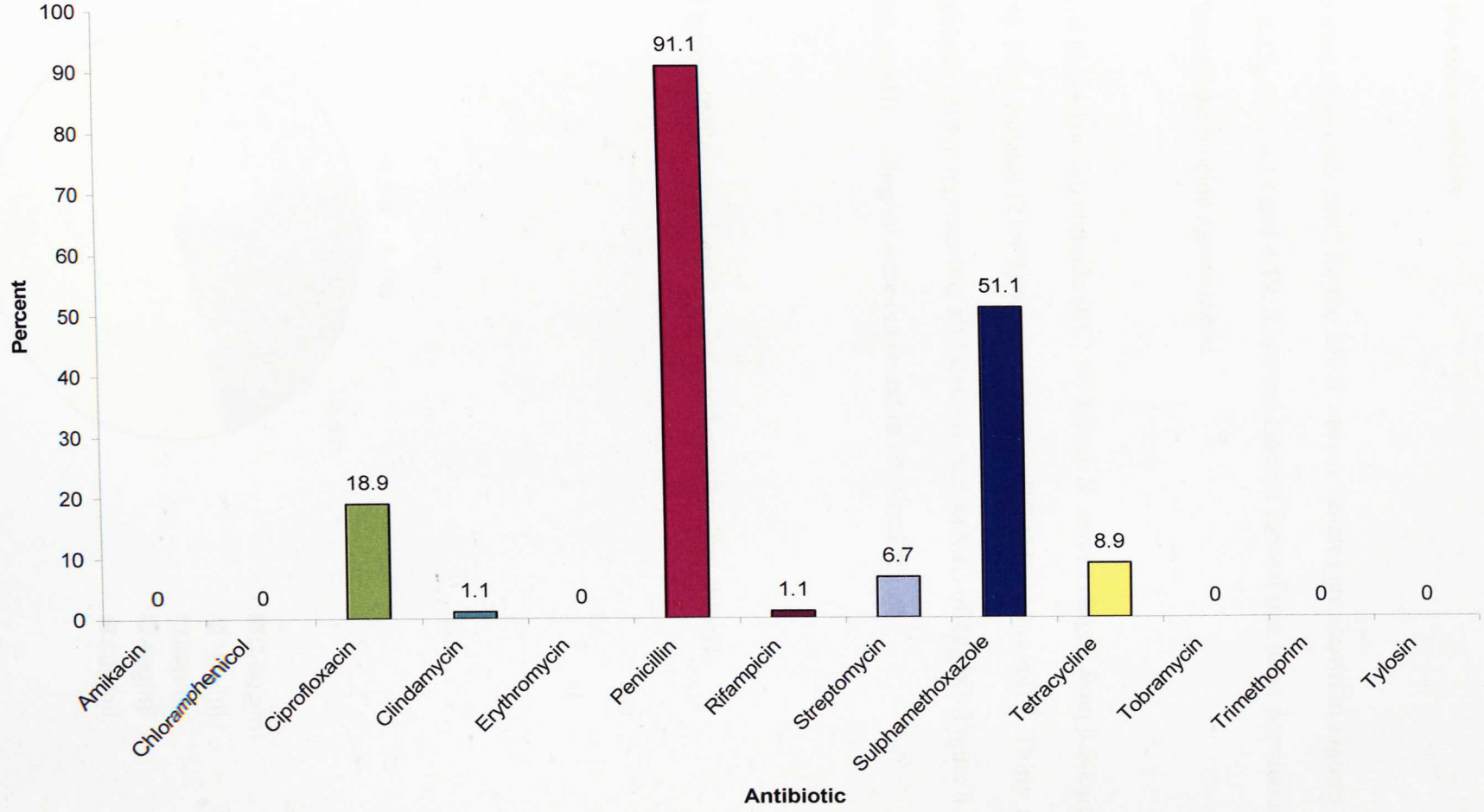


Figure 4.8 Frequency of antibiotic resistance in animal *S. aureus* isolates in Southwest Scotland



### 4.3.2 Biocide susceptibility

The distribution of biocide MIC for the 233 *S. aureus* isolates are shown in Figures 4.9, 4.11, 4.13, 4.15, 4.17, 4.18 and 4.19. *S. aureus* isolates formed one large population of susceptibilities to the biocide agents tested.

The MICs of benzalkonium chloride (BC) for animal *S. aureus* ranged from 0.5-8 μg/ml (Figure 4.9). Fifty isolates (21.4%) were resistant to BC (MIC ≥4μg/ml)[39]. Thirty nine of the 50 isolates (78%), representing all locations, had an MIC of 4μg/ml (Figure 4.10). Isolates with an MIC of 8μg/ml were not found in Highland region.

Figure 4.9 Susceptibility of animal *S. aureus* to benzalkonium chloride

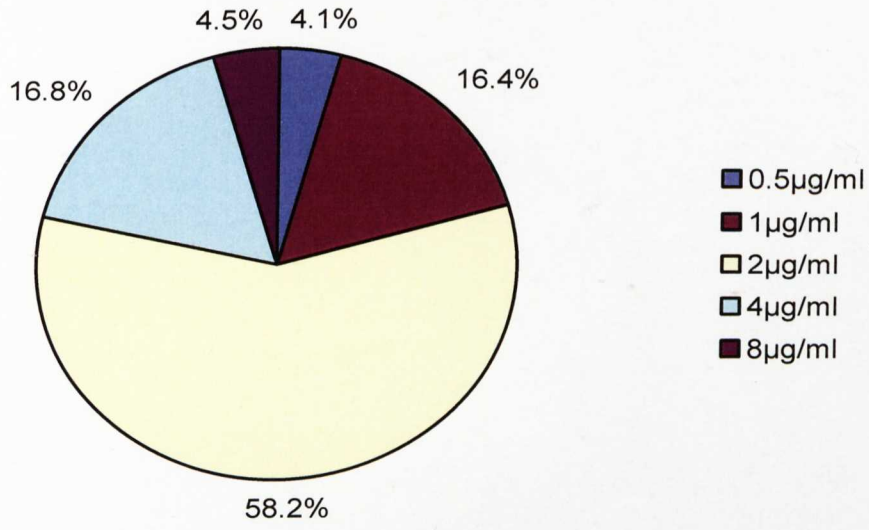
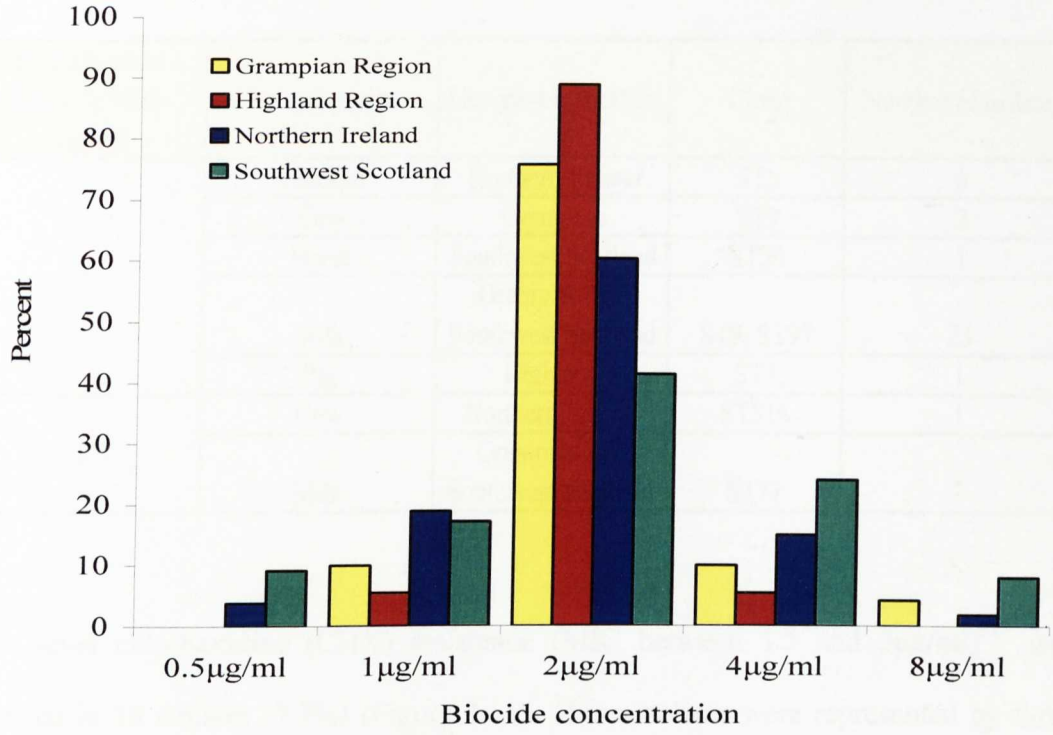


Figure 4.10 Regional variations in benzalkonium chloride MIC




Resistant isolates were detected in only five animal sources, chicken, cow, horse, milk and pig, representing seven different clones (Table 4.3). Isolates of bovine origin accounted for 69.2% with an MIC of 4µg/ml and all of the isolates with an MIC of 8µg/ml.

Table 4.3 Animal source, geographic location and clonal background of low level benzalkonium chloride resistant *S. aureus*

| Benzalkoniun chloride MIC (μg/ml) | Animal source | Geographic location | Clone | Number of isolates |
|---|---|---|---|---|
| 4 | Chicken | Northern Ireland | ST5 | 6 |
| | Cow | Grampian | ST9 | 2 |
| | Horse | Southwest Scotland | ST30 | 1 |
| | Milk | Grampian and Southwest Scotland | ST9, ST97 | 23 |
| | Pig | Highland | ST5 | 1 |
| 8 | Cow | Northern Ireland | ST816 | 1 |
| | Milk | Grampian and Southwest Scotland | ST71 | 7 |

Low level chlorhexidine (CHX) resistance (MIC between 1.5 and 3μg/ml)[397] was detected in 18 isolates (7.7%) (Figure 4.11). These isolates were represented by three animal sources and four clones (Table 4.4). No low level CHX resistance was detected in isolates from Highland region (Figure 4.12)

Figure 4.11 Susceptibility of animal *S. aureus* to chlorhexidine

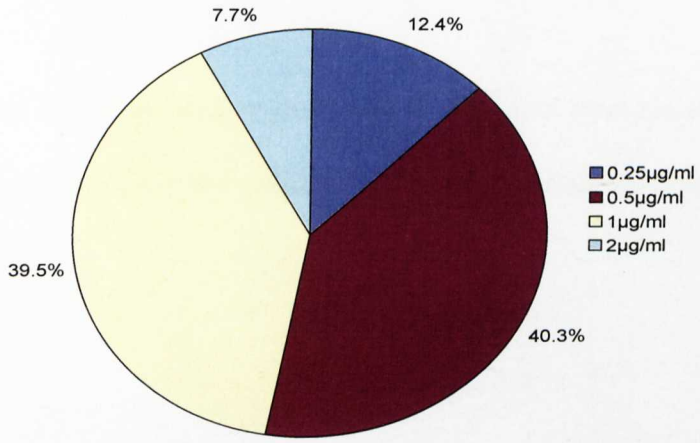
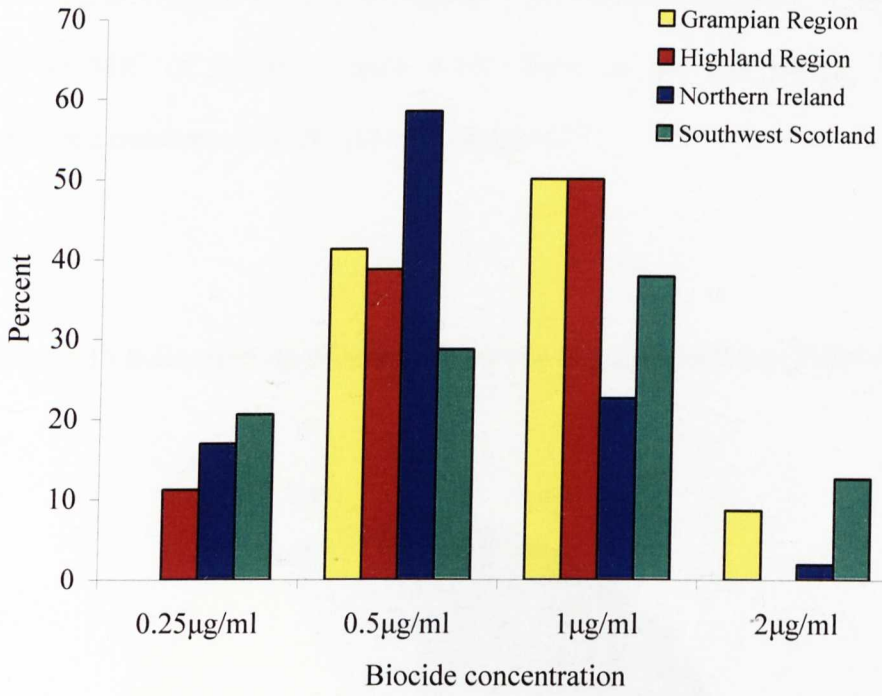


Table 4.4 Animal source, geographic location and clonal background of low level chlorhexidine-resistant *S. aureus*

| Animal source | Geographic location | Clone |
|---|---|---|
| Cow (n=1) | Northern Ireland | ST816 |
| Milk (n=16) | Southwest Scotland | ST71, ST97 |
| Turkey (n=1) | Southwest Scotland | ST5 |

Figure 4.12 Regional variations in chlorhexidine MIC




Sixteen (32%) of the 50 BC resistant isolates also showed low level resistance to CHX. All of these isolates were of bovine origin and were represented by one of only three clones (Table 4.5)

Table 4.5 Animal source, geographic location and clonal background of low level benzalkonium chloride and chlorhexidine-resistant *S. aureus*

| Animal source | Geographic location | Associated ST |
|---|---|---|
| Cow (n=1) | Northern Ireland | 816 |
| Milk (n=15) | Southwest Scotland and Grampian | 97, 9 |

The MIC range for cetylpiridinium chloride (CPC) for animal *S. aureus* isolates was 0.25-2μg/ml (Figure 4.13). Only isolates from Northern Ireland and Southwest Scotland had an MIC of 2μg/ml (Figure 4.14). None of the 233 animal *S.aureus* isolates expressed resistance to CPC (MIC = ≥4μg/ml)[416].

Figure 4.13 Susceptibility of animal *S. aureus* to cetylpiridinium chloride

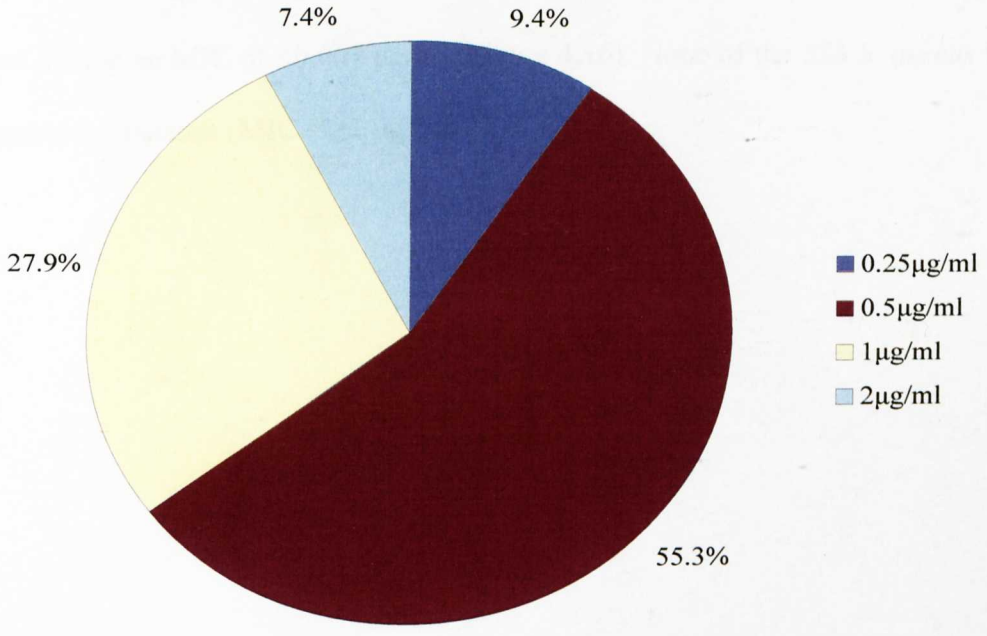
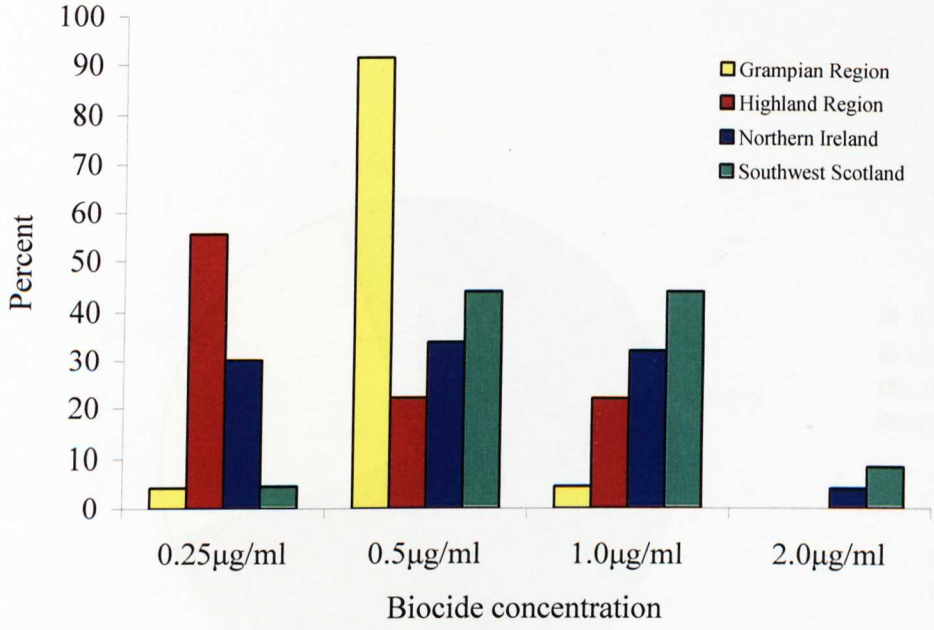


Figure 4.14 Regional variations in cetylpiridinium chloride MIC




Triclosan MICs ranged between <0.007-0.03 µg/ml (Figure 4.15). A high proportion of isolates from Highland region were from wild animals which may account for 72.2% of these having an MIC of <0.007 µg/ml (Figure 4.16). None of the 233 *S. aureus* isolates expressed resistance (MIC = ≥1 µg/ml)[417].

Figure 4.15 Susceptibility of animal *S. aureus* to triclosan

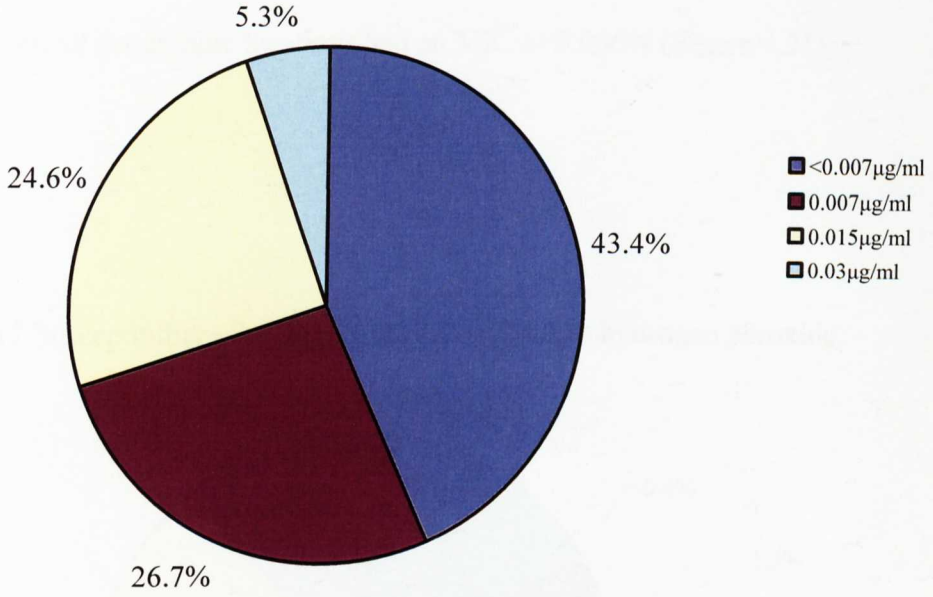
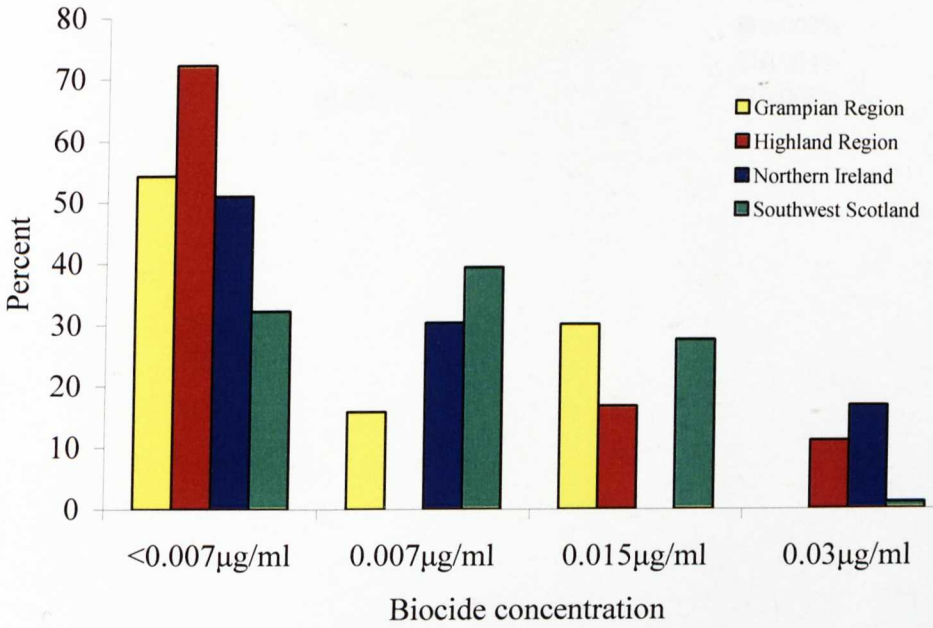



Figure 4.16 Regional variations in triclosan MIC



All *S. aureus* isolates were susceptible to $H_2O_2$, formaldehyde and copper sulphate, with MICs of 0.001-0.008% (Figure 4.17), 0.003-0.0125% (Figure 4.18), and 4-8 mM respectively (Figure 4.19)[4]. Isolates from Northern Ireland were the most susceptible to $H_2O_2$, with MICs of $\leq$ 0.004% (Figure 4.20). The majority of formaldehyde susceptible isolates from all geographic locations had an MIC of 0.006% (Figure 4.21).

Figure 4.17 Susceptibility of animal *S. aureus* isolates to hydrogen peroxide

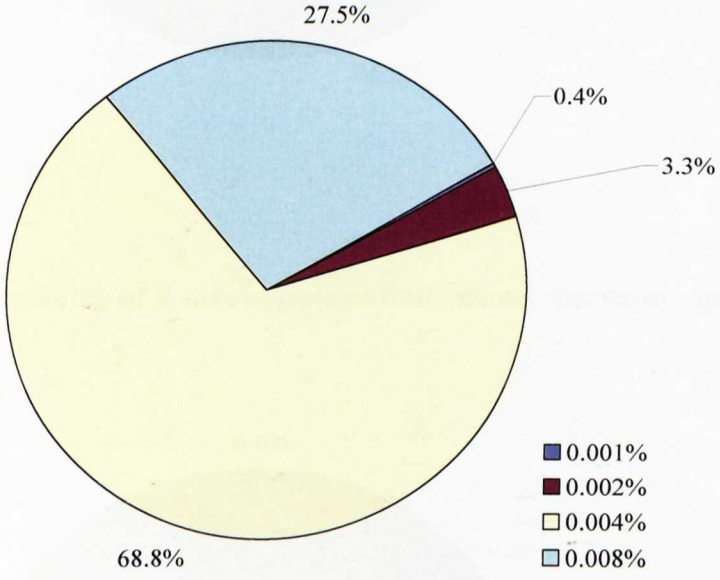


Figure 4.18 Susceptibility of *S. aureus* isolates from animal species to formaldehyde

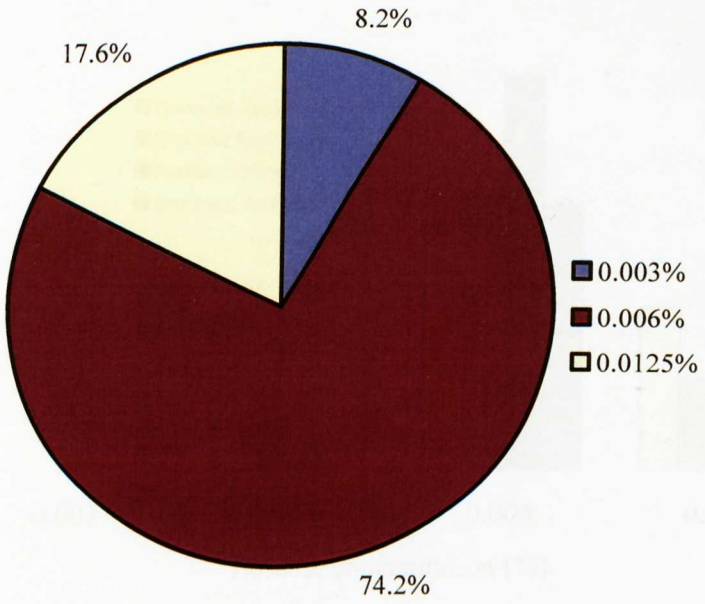



Figure 4.19 Susceptibility of *S. aureus* isolates from animal species to copper sulphate

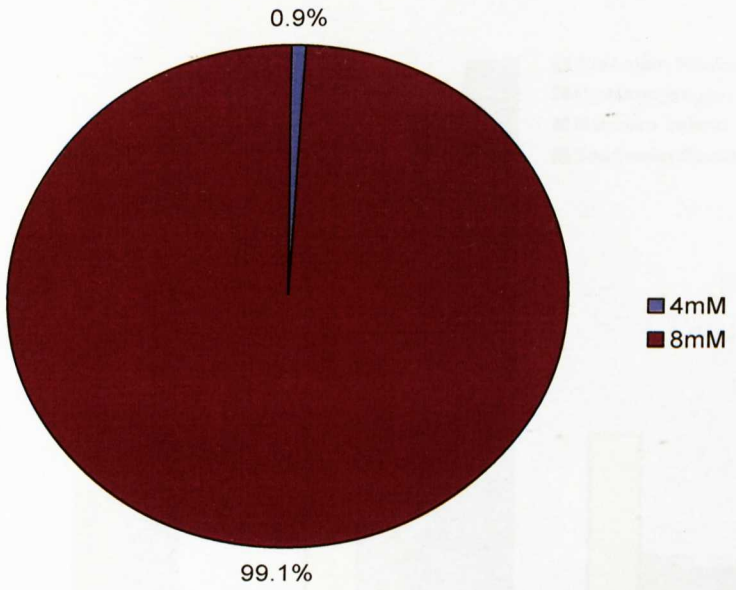


Figure 4.20 Regional variations in $H_2O_2$ susceptibility

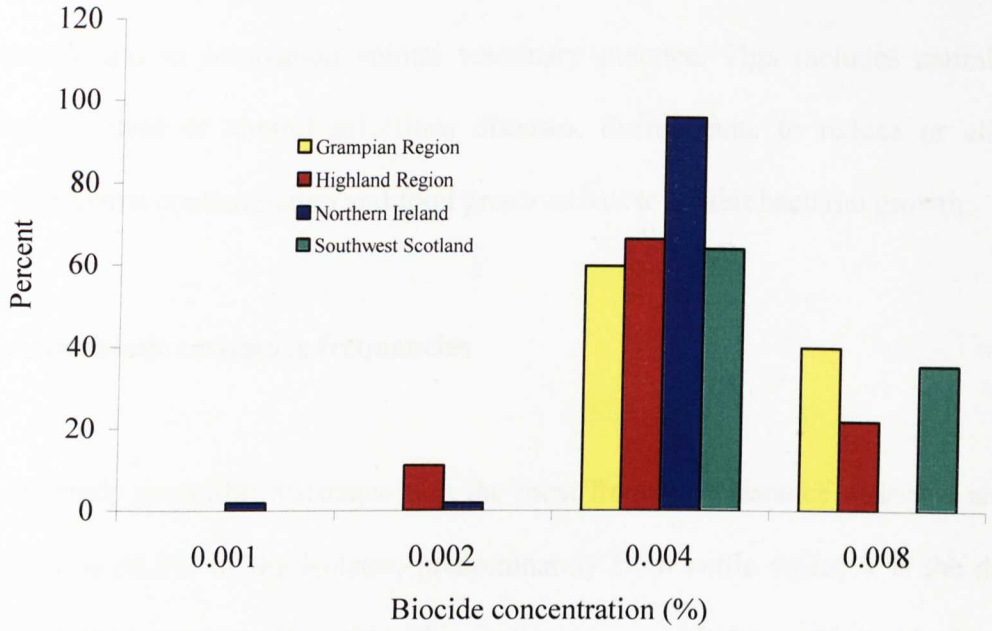
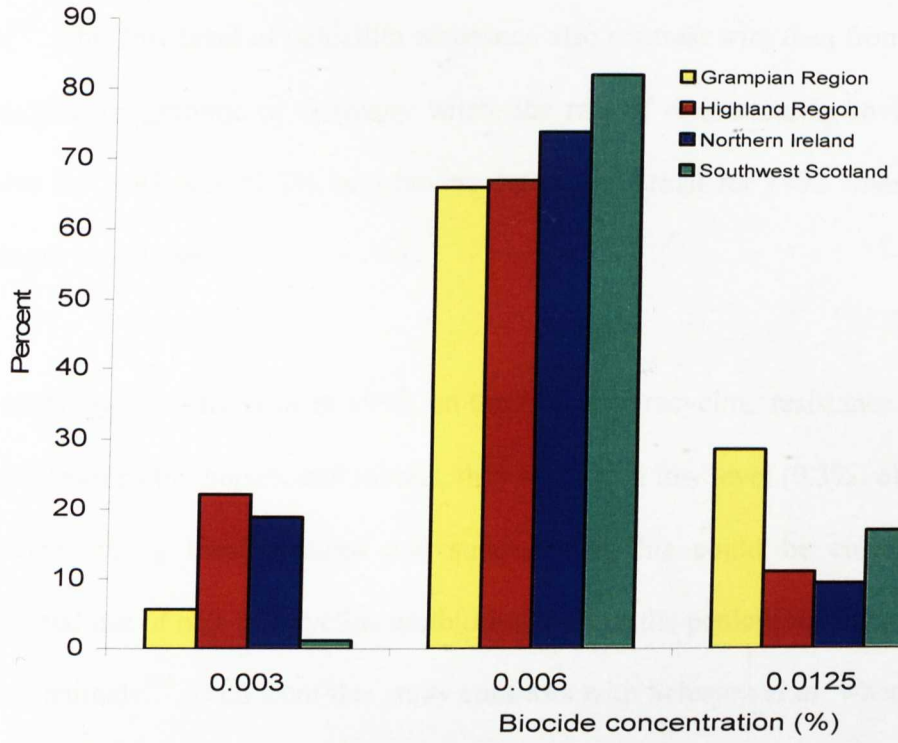



Figure 4.21 Regional variations in formaldehyde susceptibility



## 4.4 Discussion

A large number of chemical microbicidal substances are used in the production of livestock and in companion animal veterinary practice. This includes antimicrobial agents to treat or control infectious diseases, disinfectants to reduce or eliminate environmental contamination and food preservatives to inhibit bacterial growth.

### 4.4.1 Antibiotic resistance frequencies

In this study penicillin resistance was the most frequent resistance observed and was detected in 65.2% of the isolates, predominantly from cattle where it is the drug of choice in the treatment of mastitis. This finding agrees with data published by Vintov *et al*, from Ireland in 2003, where 71.4% of bovine *S. aureus* isolates exhibited penicillin resistance[458,401]. In contrast, Wagge *et al* have reported that although the rate of mastitis in cattle in Norway is high and penicillin is the antibiotic most commonly used in treatment the frequency of penicillin resistance in *S. aureus* from cattle remains low at 10%[462]. This low level of penicillin resistance also contrast with data from the national monitoring programme of Germany where the rate of resistance, in bovine *S. aureus* isolates for 1997 was 52.2%, and bovine data from Brazil for 1992 where the rate of resistance was 43.9%[476].

In a study by Schwarz *et al* in 1998, on the rate of tetracycline resistance in *S. aureus* isolated from cattle, horses, and rabbits, they reported a low level (9.3%) of tetracycline resistance among these isolates and suggest that this could be correlated to the preferential use of non-tetracycline antibiotics, such as the penicillins, to control disease in these animals[384]. Data from this study contrasts with Schwarz *et al* where the rate of

resistance in wild (Pheasant and Phillips goose hawk), and domestic animal sources (chicken, cow, milk, pig, and turkey) was almost four times higher (36.4%) and was in close accordance with data from a study by Aarestrup *et al* [5] (2000) where 47% of *S. aureus* isolated from chickens were tetracycline resistant, and Güler *et al*[169] (2005) where 27.9% of bovine *S. aureus* were tetracycline resistant.

Sulphamethoxazole in combination with trimethoprim accounted for 20% of the veterinary antibiotic sales in the UK[428]. While the overall frequency of resistance to sulphamethoxazole in this study was 23.2%, resistance to trimethoprim was found in only three isolates, two bovine and one porcine. This level of sulphamethoxazole resistance contrasts with data from Vintov *et al* who have shown a mean rate of resistance of only 3.7% among bovine *S. aureus* isolates from across Denmark, England, Finland, Germany, Iceland, Ireland, Norway, Sweden, Switzerland and the USA[458]. In contrast, Werckenthin *et al* have reported levels of resistance to sulphonamides in Denmark and Germany of 52.6% and 50% respectively among bovine *S. aureus*[476].

The veterinary fluoroquinolone enrofloxacin is commonly used in the systemic treatment of disease in a wide range of animal species[118]. This antibiotic is closely related to fluoroquinolones, including ciprofloxacin, which is an active metabolite of enrofloxacin, used in human clinical medicine[106]. In this study ciprofloxacin resistance was the fourth most common resistance, and was detected in 19.7% of the isolates. This level of resistance was comparable to data published by Aarestrup *et al,* in 2000, where the rate of resistance in *S. aureus* isolated from Danish poultry was 30%.[5] These levels of resistance contrast with German data published by Wallmann[465], from 2002-2003,

where no resistance to enrofloxacin was detected from *S. aureus* isolated from cases of respiratory disease in pigs and cattle or dairy cows with acute mastitis and by Güler *et al*, in which only 1.8% of *S. aureus* isolated from Turkish cattle with acute mastitis, from 1995-2004, exhibited resistance to enrofloxacin[169].

The macrolides, tylosin and spiramycin, were commonly used as AGP in the production of pigs and poultry until banned in the EU in 1999. Large quantities continue to be used in domestic and companion animal therapy[10]. Macrolide resistance data from CNS isolates from poultry in Switzerland, *S. hyicus* from pigs, and *S. aureus* from poultry in Denmark have all shown a much higher frequency of macrolide resistance than the 7.9%, in isolates from pheasant, pig, Phillips goose hawk and cow, detected in this study[5,144,471,476]. *S. aureus* isolated from cattle in Germany have shown a rate of resistance (11.4%) to macrolides more in line with the results in this study[476].

A low level of resistance to the aminoglycoside, other than streptomycin, has previously been reported by Frei *et al* in livestock animals[144]. In this study streptomycin resistance was found in 12.3% of isolates, mostly from cattle, which is consistent with resistance data from *S. aureus* isolated from mastitic cattle in Brazil[476], Switzerland, and England with resistance rates of 12.1%, 11.8% and 9.2% respectively[459], and rabbit and ovine data from Spain with a resistance rate of 16%[158]. In contrast, higher levels of streptomycin resistance was found in Germany where 29.3% of bovine isolates from mastitis were resistant[476].

Reports of methicillin resistance in *S. aureus* in animals have become more frequent in recent years[270,387]. As early as 1975 Devrise reported the isolation of 68 MRSA from mastitic milk samples from Belgian dairy herds[103]. More recently MRSA have been isolated from surgical wound infections in horses[467], respiratory, urinary and wound infections in dogs, cats, a rabbit and a seal[270,318]. These MRSA isolates have been shown to closely resemble the predominant clone found in humans (EMRSA-15) suggesting that the transmission of MRSA between humans and animals can and does occur[318]. Reports by van Belkum *et al*, Guardabassi *et al* and Wulf *et al* have recently confirmed pig to human transmission of MRSA ST398, a lineage thought to have originated in pigs[443,461,492]. This strain has now been detected in pig populations in numerous European countries, Canada and Singapore and represents an important human health risk[461]. However, all 233 of the *S. aureus* isolates tested from Scotland and Northern Ireland were susceptible to oxacillin.

Virginiamycin, a veterinary streptogramin, was used in Europe as an AGP until banned in 1999. Its use as an AGP in the production of chickens, turkey, pigs, and cattle continues in the USA and this has been shown to select for virginiamycin resistant strains of *E. faecium* which are cross-resistant to the human streptogramin quinupristin/dalfopristin (Synercid™)[287,427,475]. Reports of streptogramin non-susceptibility in human isolates of *S. aureus* have been published[274]. There have been no reports of quinupristin/dalfopristin resistance in animal *S. aureus* isolates, which is consistent with data from this study.

Linezolid, a recently introduced antibiotic for the treatment of multiresistant Gram positive infection in humans, has never been licensed for use in animals. There have been several reports of linezolid resistance in clinical isolates of VRE[182,327]. A review of the literature has revealed a report of linezolid resistance in three MRSA. These isolates were from patients receiving linezolid for dialysis-associated peritonitis, and exhibited a G2576T mutation in DNA encoding the central region of domain V of 23S rRNA[435]. This mechanism of resistance has only been reported in rare laboratory linezolid resistant mutants of *S. aureus*[435]. Aires *et al* have more recently shown that the presence of chloramphenicol/florfenicol (*cfr*) resistance gene encoding a 23S rRNA methyltransferase confers cross resistance to linezolid[24]. Even though linezolid is not licensed for use in animals florfenicol is frequently used in the treatment of livestock. Therefore, the generation of *cfr* mediated cross resistance to linezolid would have health implications for humans. So far there have been no reports of *S. aureus* resistant to linezolid in animals, which concurs with data from this study.

Glycopeptide resistance is a limited but growing global problem in *S. aureus* isolates from humans[67,187]. No glycopeptide resistance was detected in this collection and there have been no publications of glycopeptide resistance in *S. aureus* isolated from animals even though avoparcin, a veterinary glycopeptide, was used as a growth promoter in pig and poultry production for many years until the rise in GRE forced the EU to ban its use.

In this study 9.8% of isolates were resistant to clindamycin which was consistent with the rate of 7.7% reported by Gentilini *et al* in bovine *S. aureus* from Argentina isolated between 1996-1998[152] and 7.1% reported by Rajala-Schultz *et al* in bovine CNS from the USA isolated between 2001-2002[345], but was higher than the published resistance rates in *S. aureus* from cases of bovine mastitis in Denmark (2.3%), Switzerland (3.2%), Brazil (4.5%), and France (4%) isolated between 1994-1999[476]. The frequency of clindamycin resistance of bovine *S. aureus* from Germany, isolated in 1997, was more than double (22.8%) the rate seen in this study[476].

Resistance to mupirocin is divided into two groups: low level with MICs of >8 - ≤256 mg/l and high level with MICs >256 mg/l. Low level resistance is due to alterations in isoleucyl-adenylate-tRNA synthetase (IRS) that inhibit protein synthesis. High level resistance is associated with an additional IRS that is encoded by the *ileS2* gene commonly reported on plasmids[333]. Both high and low level mupirocin resistance has been reported in *S. aureus* isolated from humans although the frequency of resistance remains low[333]. In a study by Walker *et al* it was shown that a reduction in the prevalence of mupirocin resistance could be achieved with more restrictive controls on prescribing[464]. A survey of mupirocin susceptibility of human clinical Gram positive pathogens isolated in Korea failed to detect mupirocin-resistant staphylococci in 1999, 14 years after its introduction[251]. In a study by Yun *et al*, in 2003, they report high level mupirocin in five percent of human clinical *S. aureus* and no low level resistance detected[496]. Manian *et al* have reported the asymptomatic nasal carriage of a mupirocin-resistant MRSA in a pet dog that was associated with recurrent infections and colonisations among household contacts[280]. This finding was, however, considered transmission from a human source. The observation that no mupirocin resistant isolates

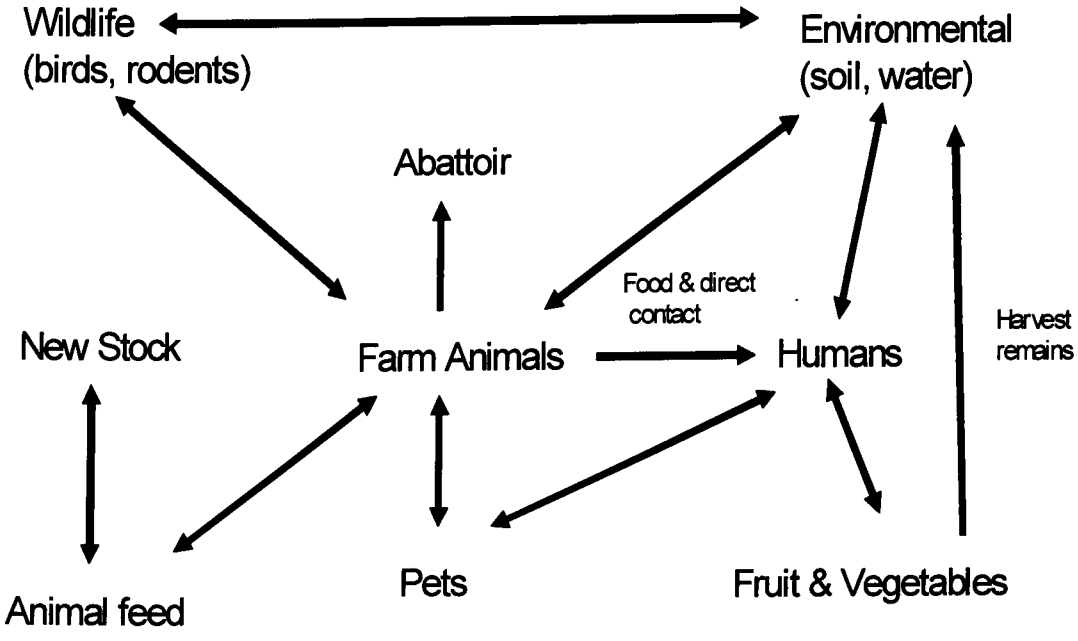
were detected in this study probably reflects the exclusive use of this drug in human medicine.

The frequency of antibiotic resistance varied geographically with only 50% of *S. aureus* from Highland region resistant to one or more antibiotics while the frequency was highest in Southwest Scotland (95.6%). Werckentin *et al* have also reported that the rates of resistance of animal staphylococci varied widely according to the animal species, resistance properties and geographic source of the isolates[476]. Resistance to only four of the 22 antibiotics tested: ciprofloxacin, clindamycin, penicillin and tetracycline were detected in all locations. The highest frequency of resistance to ciprofloxacin and tetracycline and lowest frequency of penicillin resistance was seen in isolates from Northern Ireland. Over 90% of these isolates were from chickens. The high frequency of resistance to tetracycline in poultry isolates in this study contrasts with the lower levels reported in broiler poultry isolates of *S. aureus* by Aarestrup *et al* [5] and CNS by Frei *et al*[144] and cattle, horse, cat, and rabbit isolates of *S. aureus* by Schwarz *et al*[384]. A study by Wallmann *et al* has also shown a high prevalence of ciprofloxacin and tetracycline resistance among *S. aureus* from poultry. They concluded that apart from ciprofloxacin and tetracycline, antibiotic resistance could still be considered low among poultry *S. aureus*[465]. This was the case for *S. aureus* isolates from Northern Ireland. However, approximately 20% of poultry isolates from Northern Ireland were also resistant to penicillin. Tetracycline resistance was also high among bovine isolates from Grampian region (40.3%) and porcine isolates from Highland region (33.3%). Schwarz *et al* have also shown staphylococci from pigs to have a high rate of tetracycline resistance[384]. The frequency of resistance among bovine isolates in that study was only 1%. They concluded that the high level of tetracycline resistance in porcine *S. aureus* could be related to the widespread use of tetracycline on pig farms

and the low levels of tetracycline resistance in bovine *S. aureus* could be correlated to the preferential use of non-tetracycline antibiotics such as the β-lactams[384]. Certainly, among the *S. aureus* isolates from bovine sources, mainly from Grampian region and Southwest Scotland, the levels of penicillin were high. Similarly high levels of penicillin resistance have been found among staphylococci from domestic animals in a number of different countries ranging from 50% in the USA to 71.4% in Ireland[458]. In the Scandinavian countries, where the use of penicillin in cattle and poultry is tightly controlled the rates of resistance to penicillin were much lower[5]. In this part of the study resistance levels for clindamycin were higher than for erythromycin, across the four locations. As clindamycin, a lincosamide antibiotic, and erythromycin, a macrolide antibiotic, have common targets in the bacterial ribosome; isolates that are resistant to one class may be resistant to the other class, and to type B streptogramins ($MLS_B$ phenotype). Luthje *et al* have reported clindamycin resistant erythromycin susceptible staphylococci isolated from animals. These isolates carried copies of the *lnu* (A) gene that encodes a lincosamide inactivating enzyme with no activity against macrolide antibiotics[278]. It is possible that the higher frequency of clindamycin resistance among the isolates in this study could be due by the presence of the *lnu* (A) gene. Resistance to clindamycin varied from 1.1% in Southwest Scotland to 27.8% in Highland region. High levels of clindamycin resistance in *S. aureus* from cattle have been reported from Finland, Germany, Iceland, Ireland, Switzerland and the USA[97]. While giving no exact figure on the percentage frequencies of resistance in these countries the reported $MIC_{90}$ in each country was between 4-8 μg/ ml (resistance breakpoint >0.5 μg/ml[20]). In contrast, no resistance to clindamycin and only 3% resistance to erythromycin was reported by Gianneechini *et al* among staphylococci from dairy cattle in Uruguay[154].

It is interesting to note that an isolate from a Phillips goose hawk from Northern Ireland, a wild bird which is unlikely to have received sustained antibiotic therapy, was multiply antibiotic resistant exhibiting resistance to five antibiotics while the isolate from a saker hawk was fully susceptible. Numerous sources of antibiotic contamination, antibiotic resistant organisms and resistance genes have been reported and it is possible that this multi-resistant isolate in the wild bird may have originated from one of these environmental sources or through feeding on contaminated rodent pests from farms (Figure 4.22)[336,426].

Figure 4.22 Model of the distribution and transfer of antibiotics, antibiotic resistance genes, and bacteria in the biosphere[426].



### 4.4.2 Biocide MICs

The most commonly used disinfectants for veterinary purposes are formaldehyde, iodine, chloride compounds and copper sulphate. Copper sulphate is also used as a mineral supplement in pigs, poultry and cattle[4]. Resistance to quaternary ammonium compounds (QAC) is well known and several resistance genes have been identified in both Gram positive and Gram negative organisms. Recently Hasman and Aarestrup have identified a transferable copper resistance gene in enterococci from animals and humans[176].

Data on the susceptibility of *S. aureus* isolated from animals to antimicrobial compounds used for disinfection is limited. This aim of this study was to determine the frequency of biocide and copper sulphate resistance among *S. aureus* isolates isolated from animals.

The results presented in this study indicate that there has been no development of resistance to, copper sulphate (≥16 mM)[4], cetylpiridinium chloride (≥4 μg/ml)[40], hydrogen peroxide, formaldehyde, or triclosan (>0.1 μg/ml)[417] as all isolates formed one large population in their susceptibilities to these compounds[4].

Resistance to cationic biocides in *S. aureus* is plasmid mediated and is due to the QAC series of proteins. These resistance genes have been shown to be located on β-lactamase plasmids and are also known to integrate into the chromosome[397]. No clear-cut definition exists for the classification of staphylococci as QAC-susceptible or QAC-resistant isolates and the testing methodologies used are not standardised. In a study on *S. aureus* carrying the *smr* gene; McDonnel and Russell classified isolates as BC

resistant with an MIC >3 μg/ml. Leelaporn *et al* reported the MIC of BC resistant *S. aureus* to be between 4 and 6 μg/ml[255] and in another study Littlejohn *et al* found the MIC to be 6 μg/ml[267]. In contrast, Bjorland *et al* have reported the MIC of *S. aureus* carrying the *smr* gene to be between 2.5 and 3 μg/ml[40]. In this part of the study a concentration of 4 μg/ml for BC was used for the detection of QAC-resistant *S. aureus* isolates. Fifty (21.4%) isolates were resistant to BC. These isolates were from multiple animal sources and a variety of clonal backgrounds. In a study by Aarestrup *et al* 23% of *S. aureus* isolated from livestock had an MIC to BC of ≥4 μg/ml. In that study no resistance cut-off point was given and all isolates were considered as susceptible[4]. The rate of resistance in this study is over twice that reported by Bjorland *et al* from dairy cattle[40]. A study by Fraise, on the susceptibility of hospital strains of EMRSA 16 to BC reported MICs of 0.08 μg/ml, which is 6 times lower than the lowest MIC of *S. aureus* isolates in this study. Benzalkonium chloride resistant *S. aureus* isolates occur frequently in animals, especially dairy cattle, in the UK. This high rate of resistance may be a reflection of the long term use of QAC in the treatment of bovine mastitis.

The MIC values to chlorhexidine obtained in this study were in the range 0.25-4 μg/ml. Low level resistance to CHX has been determined as having an MIC between 1.5 and 3 μg/ml[416]. Eighteen isolates (7.7%) exhibited low level CHX resistance (MIC 2 μg/ml). Data published by Pitt *et al*[339], on CHX susceptibility of human *S. aureus*, and Aarestrup *et al*[4], on CHX susceptibility of animal *S. aureus*, have shown very similar CHX MIC values to those obtained in this study. However, the MICs of isolates in this study were spread over a wider range.

Sixteen of the 50 BC resistant isolates also exhibited low level resistance to CHX. A similar frequency of co-resistance between BC and CHX has been reported by Sidhu *et al* in an investigation into the genetic linkage between QAC resistance genes and β-lactamase resistance among human clinical staphylococcal isolates[397].

Reduced susceptibility to CPC in MRSA has been reported by Irizarry *et al*[198]. Over 90% of the MSSA isolates in that study were susceptible to CPC at concentrations of < 2μg/ml. A similar high frequency of low level CPC resistance has been reported in human MRSA, compared with human MSSA, by Suller *et al*[416]. All isolates in this study were susceptible to CPC with MICs in the range 0.25 to 2μg/ml.

*S. aureus* strains have emerged that exhibit low-level resistance to triclosan (MIC 1-4 μg/ml)[79,369,441]. Cookson *et al* have reported low level triclosan resistance (MIC 2-4 μg/ml) in human MRSA strains. Bamber and Neal have also reported high level triclosan resistance in human hospital *S. aureus* strains (MICs up to 32 μg/ml). All isolates in this study had MICs to triclosan in the range <0.007-0.03μg/ml with over 70% susceptible at MICs of 0.007 μg/ml or less. These data concur with results published by Suller *et al*[416] who failed to detect triclosan resistance among human clinical *S. aureus*. They question the clinical significance of low-level triclosan resistance detected by others in *S. aureus*. Triclosan is known to be stable in the environment and may be present on surfaces at low concentrations when used as a disinfectant. There is concern that continuous exposure to sub-MIC concentrations of triclosan may give rise to organisms with increased resistance to disinfectants and antibiotics, although this concept remains to be demonstrated[417]. Suller and Russell attempted to select for biocide resistant *S. aureus* isolates by repeated exposure of the

isolates to increasing concentrations of biocide. They were able to demonstrate increased MICs to CHX, CPC and triclosan. In the absence of biocide, however, the low level resistance was not stably maintained[416]. Work carried out by Fitzgerald *et al* also failed to produce stably maintained biocide resistance in *E. coli* and *S. aureus*[138]. Nicoletti *et al* were able to demonstrate high level stably maintained resistance to CHX and QACs in *Pseudomonas aeruginosa* and *Serratia marcescens*[313]. Suller *et al* concluded that there was no evidence to suggest that staphylococcal strains will show increased resistance in the human clinical environment despite the potential for repeated exposure to high concentrations of biocide[416]. In contrast, Bjorland *et al* have shown the presence of QAC resistant *S. aureus* from dairy cattle that had persisted for over 20 months and were thought to be as a consequence of the continuous use of teat cream containing QAC[40]. *S. aureus* isolates in this study were collected between 1999 and 2001 and were continuously subcultured on non-selective media over a period of four years prior to biocide susceptibility testing. Therefore, low level resistance among these isolates has remained stable in the absence of biocide exposure over an extended period.

Early studies into the bactericidal effectiveness of $H_2O_2$ against *S. aureus* strains have been shown to be dependent upon a number of test parameters such as $H_2O_2$ concentration and temperature and the inherent characteristics of the test strain[460]. The bactericidal effect of hydrogen peroxide was shown to be less effective at an incubation temperature of 37°C than at 54°C. The increased resistance to hydrogen peroxide is thought to be related to the variability of catalase activity between *S. aureus* strains[17]. Amin *et al* were able to show that *S. aureus* strains from cattle with high catalse activity were more resistant to the effects of $H_2O_2$.[18] These strains exhibited a two fold increase in hydrogen peroxide MIC (0.025 to 0.05%) compared to those strain with low catalase

activity[17]. All *S. aureus* isolates in this study exhibited MICs to $H_2O_2$ in the range 0.001 to 0.008% and were considerably lower than those published by Amin *et al* and Von Ruden *et al*[17,460]. The $H_2O_2$ MIC values obtained in this study are very similar to those published by Aarestrup *et al* who considered their isolates to be very susceptible[4]. Rodgers *et al* have also shown peroxide based disinfectants to be effective against *S. aureus* contamination of the surface of eggs supplied to hatcheries at the manufacturers recommended concentration[361].

Formaldehyde is a widely used disinfectant in the production of food animals. There have been few studies examining the susceptibility of *S. aureus* strains to this agent. Plasmid mediated transmissible formaldehyde resistance has been found in *Serratia marcescens* and *E. coli*[219,244]. In these studies isolates were considered resistant at an MIC value of $\geq$ 0.07%. The MIC range for isolates in this study was between 0.003 and 0.0125%. A similar range of formaldehyde MIC (0.003-0.006%) has been reported by Aarestrup *et al* among *S. aureus* isolates from domestic animal species which they considered susceptible based on the data published by Kaulfers *et al* and Kummerle *et al*[219,244].

Copper sulphate ($CuSO_4$) is commonly used as a footbath in milking yards to treat lameness in dairy cattle and as a mineral supplement in pig and poultry production[48]. Acquired and transferable copper resistance has been most thoroughly examined in Gram negative bacterial systems such as *pco* genes from *E. coli* [53]and the *cop* genes from *Pseudomonas spp*[77]. Until recently few cases of copper resistance in Gram positive bacteria have been reported and in most cases the resistance mechanism remains unknown. Hasman *et al* have reported the first plasmid located, transferable,

copper resistance gene in Gram positive bacteria named transferable copper resistance homologue to *copB* (*tcrB*). This plasmid was detected in an *E. faecium* isolated from a pig[176]. Few reports have been published on the susceptibility of *S. aureus* from animals to copper. In this study all isolates formed one large population of susceptibility with 95% of isolates exhibiting an MIC of 8mM. The MIC values obtained in this study are comparable to those previously observed by Aarestrup *et al*[4].

In conclusion, penicillin, tetracycline, sulphamethoxazole, ciprofloxacin and streptomycin were the most frequently detected antibiotic resistances among *S. aureus* isolates from animals. β-lactam antibiotics, including penicillin, ampicillin, amoxicillin and hetacillin, are the most widely used class of antimicrobial agents used in the treatment of disease in cattle. The high frequency of penicillin resistance in this study may be a reflection of the large proportion of isolates tested (67%) for antimicrobial susceptibility being of bovine origin. No resistance was detected against fusidic acid, gentamicin, kanamycin, linezolid, mupirocin, oxacillin, quinupristin/dalfopristin, teicoplanin or vancomycin. Resistance to all other antibiotics was low. The lack of resistance to linezolid and mupirocin in *S. aureus* from animals is most likely due to their exclusive use in human clinical medicine.

Regional variations in the frequency of antibiotic resistance in *S. aureus* from animals have been shown previously and were also apparent in this study[458]. Penicillin and tetracycline resistance was predominant in Grampian and Highland regions, ciprofloxacin and tetracycline resistance in Northern Ireland and penicillin and sulphamethoxazole resistance in Southwest Scotland. Variations in the distribution and frequency of antibiotic resistance across different locations can be attributed to differences in the regional prescribing policies of veterinary practitioners.

Extensive use of disinfectants has led to the selection of staphylococcal isolates resistant to such compounds in human hospital environments, as well as in food animal production[179,255]. In the present study *S. aureus* isolates from livestock, wild and companion animals have either not or only to a limited degree developed low level resistance to biocides. The failure of Suller *et al* to train *S. aureus* isolates with stably maintained resistance to biocides casts doubt over the clinical significance of biocide resistance. It has been shown that there can a linkage between biocide and antibiotic resistance in staphylococci isolated from humans[397,417]. Disinfectants may therefore act as selective pressures for the retention of plasmids that contain the genes for resistance to numerous antimicrobial substances. Further work needs to be done investigate the genetic basis of low level resistance to BC and CHX and determine whether there is any genetic linkage between biocide and antibiotic resistance in the *S. aureus* isolated from animals from the UK[397].

# Chapter 5 Molecular mechanisms of tetracycline and erythromycin resistance and horizontal resistance gene transfer studies

## 5.1 Introduction

Antimicrobial substances are vital drugs for prophylaxis and treatment of bacterial infections in humans and animals[144]. Since the introduction of antimicrobials into human medicine, staphylococci have shown a frequent and rapid development and spread of antimicrobial resistance, particularly in nosocomial infections. Unfortunately, this development has not been documented continuously in the veterinary field[476]. In Europe, staphylococci isolated from animals are included in the national antibiotic susceptibility monitoring programmes of eight countries (Austria, Belgium, Denmark, France, Germany, the Netherlands, Portugal, and the UK). Schwarz and co-workers and Aarestrup and co-workers have determined the presence and frequency of selected antibiotic resistance genes among staphylococci isolated from animals from across Europe and in particular Germany and Denmark respectively[5,382,384].

Tetracyclines and macrolides have been used for therapy and prevention of staphylococcal infections in humans, animals, and plants[384]. Studies on the genetic basis of macrolide and tetracycline resistance in staphylococci of human origin have been performed[311]. In addition, the distribution of tetracycline and macrolide resistance determinants among *Staphylococcus* spp, including *S. aureus*, from several domestic animal species has previously been reported from selected countries[5,263,384,454]. An investigation of the current literature has no reports on the genetic basis of tetracycline or macrolide resistance in *S. aureus* from livestock, wild, or companion animals from the UK and only limited data from other countries including the USA[46,269,310].

The increase in antimicrobial resistance and the impact on human health is an emerging problem worldwide. Since exposure to antimicrobial agents is the most important factor in the development of antimicrobial resistance, animals and animal products, including food, are thought to be significant sources of resistant bacteria for humans[325]. *S. aureus* is becoming increasingly virulent and resistant to antibiotics due to the horizontal transfer of mobile genetic elements (MGE)[418,463]. The transfer of antibiotic resistance genes between a wide range of bacterial species, in human and laboratory rodent intestine, between animal and human strains of enterococci, and other bacterial species has been investigated[27,89,96,394].

The aims of this study were to determine the presence and relative frequency of genes encoding tetracycline, and macrolide resistance in *S. aureus* from livestock, wild, and companion animals and humans from the UK and companion animals from the USA; to investigate possible structural variation in the *tet*K genes from *S. aureus* isolates from animals and humans; and to investigate the transferability of *tet*K resistance plasmids from animal strains to the lab strain *S. aureus* 8325-4.

## 5.2 Materials and methods

### 5.2.1 Bacterial strains

Four isolate collections were used; the first isolate collection comprised of 74 tetracycline resistant and 16 erythromycin resistant animal *S. aureus* isolates (Table 5.1). These resistant isolates were identified amongst the 233 animal *S. aureus* collection (Table 2.1), the second isolate collection comprised of seven tetracycline and 12 additional erythromycin resistant animal MRSA isolates from the USA, supplied by the Dept of Veterinary Medicine, University of Pennsylvania (Table 5.2), the third isolate collection comprised of 53 tetracycline resistant human MSSA and MRSA isolates (Table 5.3) and the fourth isolate collection comprised of 67 erythromycin resistant human MRSA isolates (Table 5.4). Isolate collections three and four were supplied by the Scottish MRSA reference laboratory.

Table 5.1 Tetracycline and erythromycin resistant animal *S. aureus* isolates from the UK

| Animal species | | Tetracycline resistant isolates | Erythromycin resistant isolates |
|---|---|---|---|
| Livestock | Chicken | 30 | |
| | Cow | 28 | 10 |
| | Milk | 7 | |
| | Pig | 5 | 3 |
| | Turkey | 2 | |
| Wild | Pheasant | 1 | 2 |
| | Phillips goose hawk | 1 | 1 |
| Total | | 74 | 16 |

Table 5.2 Tetracycline and erythromycin resistant animal *S. aureus* isolates from the USA

| Animal species | USA | |
|---|---|---|
| | Tetracycline resistant | Erythromycin resistant |
| Dog | | 7 |
| Horse | 6 | 4 |
| Parrot | 1 | 1 |

Table 5.3 Tetracycline resistant human MSSA and MRSA isolates

| Isolation year | PFGE type | MSSA | MRSA |
|---|---|---|---|
| 2001 | E15 | | 1 |
| | E16 | 2 | 4 |
| | 104 | 5 | 3 |
| | 105 | 2 | 2 |
| | 105/112 | | 1 |
| | 107 | 1 | |
| 2002 | 108 | | 2 |
| | 109 | | 2 |
| | 111 | 1 | 1 |
| | 112 | | 1 |
| | 116 | | 3 |
| | 117 | | 1 |
| 2003 | 118 | | 1 |
| | 120 | | 1 |
| | 121 | 1 | 1 |
| | 122 | | 1 |
| | 124 | 1 | 1 |
| | 126 | 1 | 1 |
| 2004 | 127 | 5 | |
| | 136 | | 1 |
| | 141 | | 1 |
| | 147 | | 1 |
| 2005 | 153 | | 1 |
| | 154 | | 1 |
| | 155 | 1 | 1 |
| Total | | 20 | 33 |

Table 5.4 Erythromycin resistant human MRSA isolates

| Isolation year | PFGE type | Isolation year | PFGE type |
|---|---|---|---|
| 1997 | 108 | 2002 | 108-51(LD) |
| | 104 | | 112y |
| | 105a | | 126a |
| | 112a | | 15a |
| 1998 | 105h | | 16l |
| | 111b | | 112 |
| | 16-28 | 2004 | 126 |
| | 99b | | 139/142 |
| 1999 | 105s | | 107/111 |
| | 108-28 | | 136 |
| | 109a | | 126 |
| | 118b | | E15 |
| | 12a | | 116/124 |
| | 15-30 | | 116/124 |
| | 16-108 | | 127 |
| | 99a | | 127 |
| 2000 | 128 | | 138 |
| | E17 | | E16 |
| | 108qr | | 116/124 |
| 2001 | 105-50 | | E15 |
| | 108-42 | | |
| | 111t | | E16 |
| | 112w | | |
| | 111 | | 105/108 |
| | 125a | | |
| | 126c | | 105/108 |
| | 140a | | |
| | 141a | | 139/142 |
| | 145a | | |
| | 149a | | 132/154 |
| | 16-119 | | |
| | CNS3 | | |

### 5.2.2 *tet*K long PCR primer design

Primers were designed from Genebank sequence S67449, for the *tet*K gene of plasmid pT181, using *Genefisher* interactive primer design software http://bibiserv.techfak.uni-beilefeld.de/bibi/Tools_Primer_Design.html, and amplified an 1146bp internal fragment of the 1380bp *tet*K gene.

### 5.2.3 Resistance gene PCR

The genes coding for *ermA*, *ermB*, *ermC*, *tet*K, *tet*L, *tet*M and *tet*O were amplified using the PCR primers and amplification conditions outlined in Table 5.5. PCR was performed on a Techne Genius thermal cycler. A positive and negative control was included in each run.

### 5.2.4 Gel electrophoresis

Electrophoresis was performed using six microlitres of PCR product loaded in a 1.5% Neuseive agarose (Cambrex, UK) gel in a 0.5% TBE solution (Gibco, UK) at 150V for 40 minutes.

Plasmid DNA electrophoresis was performed using thirty microlitres of plasmid extract loaded in a 0.8% Neuseive agarose (Cambrex, UK) gel in a 0.5% TBE solution (Gibco, UK) at 65V for 20 minutes followed by 23V for 16 hours. The size of each plasmid was determined by comparison with a λ DNA molecular weight ladder (0.12-23.1kb) (Roche Applied Sciences, Germany).

Following electrophoresis gels were stained with 1μg/mL ethidium bromide solution (Sigma, UK) for 30 minutes visualised by uv transillumination (UVP, UK) and the image stored digitally using VisionWorks 32 software. The size of each PCR product was determined by comparison with the appropriate control strain and an appropriate molecular weight ladder (Gibco, UK).

Table 5.5 PCR primers

| Primer | Product (bp) | Primer sequence (5'-3') | Positive control strain | PCR reaction mix and conditions | Reference |
|---|---|---|---|---|---|
| ermA-1 | 190 | AAGCGGTAAACCCCTCTGA | 2387/00 | Multiplex PCR[a] Initial denature 94°C 4 minutes, 30 cycles of 94°C 30 seconds,55°C 30 seconds, and 72°C 30 seconds, final extension 72°C 4 minutes | Lina *et al*[25] and Strommenger *et al*[49] |
| ermA-2 | | TTCGCAAATCCCTTCTCAAC | | | |
| ermC-1 | 299 | AATCGTCAATTCCTGCATGT | 113ero-2222 | | |
| ermC-2 | | TAATCGTGGAATACGGGTTTG | | | |
| ermB-1 | 359 | CCGTTTACGAAATTGGAACAGGTAAAGGGC | RN11 | PCR-1[a] Initial denature 94°C 3 minutes, 30 cycles of 94°C 30 seconds, 55°C 30 seconds, and 72°C 1 minute, final extension 72°C 4 minutes | Lina *et al*[25] |
| ermB-2 | | GAATCGAGACTTGAGTGTGC | | | |
| tetK-1 | 169 | TCGATAGGAACAGCAGTA | pT181 | Multiplex PCR[a] Initial denature 94°C 5 minutes, 35 cycles of 94°C 1 minute, 55°C 1 minute, and 72°C 1 minute 30 seconds, final extension 72°C 4 minutes | Ng *et al*[32] |
| tetK-2 | | CAGCAGATCCTACTCCTT | | | |
| tetL-1 | 267 | TCGTTAGCGTGCTGTCATTC | pSTS-9 | | |
| tetL-2 | | GTATCCCACCAATGTAGCCG | | | |
| tetM-1 | 406 | GTGGACAAAGGTACAACGAG | 2567 | | |
| tetM-2 | | CGGTAAAGTTCGTCACACAC | | | |
| tetO-1 | 515 | AACTTAGGCATTCTGGCTCAC | BM2509 | | |
| tetO-2 | | TCCCACTGTTCCATATCGTCA | | | |
| tetK-154 | 1146 | ACTGGGTAAACACTGCA | | PCR-2[a] Initial denature 94°C 10 minutes, 30 cycles of 94°C 1 minute, 55°C 1 minute, and 72°C 1 minute 30 seconds, final extension 72°C 10 minutes | |
| tetK-1300 | | AAGGATAGCCATGGCTA | | | |

[a] PCR reaction mixes are described in Table 3.1

### 5.2.5 Restriction endonuclease digestion of *tet*K long PCR product

The 1146bp predicted PCR product was fed through restriction analysis software at http://www.ualberta.ca/~stothard/javascript/index. Results indicated that endonucleases *Alu*I, *Hinc*III, *Kpn*I and *Rsa*I would give a selection of distinct restriction patterns (see Table 5.6). Endonuclease digest reactions were performed following the method in section 3.2.4.

Table 5.6 Restriction endonucleases

| Restriction enzyme | Supplier | Enzyme concentration | REact® buffer | Cleavage site | Cut positions |
|---|---|---|---|---|---|
| *Alu* I | Invitrogen, UK | 500u | 1 | AG / CT | 113, 232, 292 and 664 |
| *Hinc* II | Invitrogen, UK | 300u | 4 | GTY / RAC | 26 and 896 |
| *Kpn* I | Invitrogen, UK | 2000u | 4 | GGTAC / C | 418 |
| *Rsa* I | Invitrogen, UK | 1000u | 1 | GT / AC | 182 and 416 |

### 5.2.6 Preparation of antimicrobial stock solution for broth microdilution minimum inhibitory concentration (MIC)

Antimicrobial stock solution of 1280µg/ml was prepared using the following formula taking into consideration the assay potency of the antimicrobial.

$$\text{Volume (ml)} = \frac{\text{Actual weight of antimicrobial powder (mg)} \times \text{Antimicrobial potency (µg/ml)}}{\text{Desired concentration (1280µg/ml)}}$$

Antimicrobials were dissolved in an appropriate solvent and stored at -20°C until required.

### 5.2.7 Broth microdilution MIC

Broth microdilution MIC determination was performed following the recommendations of the British society for antimicrobial chemotherapy[21]. One hundred microlitres of Müller Hinton II broth (Becton Dickinson, UK) was added to 12 wells of a 96 well microtitre plate (Corning Incorporated, New York, USA). A 1:5 dilution of antimicrobial stock solution was performed giving a working concentration of 256μg/ml. One hundred microlitres of antimicrobial working solution was added to the first well followed by serial two-fold dilutions giving a concentration range of 128 – 0.06μg/ml. Test isolate suspension was prepared in sterile distilled water, adjusted to McFarland 0.5 (1-2 x $10^8$ cfu/ml) followed by a 1:10 dilution (1 x $10^7$ cfu/ml). Five microliters of isolate solution was added to each well (final cell concentration 5 x $10^5$ cfu/ml) and incubated at 37°C for 16-24 hours. The MIC was recorded as the lowest concentration of antimicrobial agent that completely inhibits growth.

### 5.2.8 Double disc diffusion test

All erythromycin resistant isolates were tested for inducible clindamycin resistance by the double disc diffusion method as described by Feibelkorn *et al*[130]. Isolates were inoculated on Müller-Hinton II agar following the method in section 4.2.2. The induction test was performed by manually placing a 2μg clindamycin disc and a 15μg erythromycin disc approximately 12mm apart. Each plate was incubated at 37°C in air for 16-18 hours and zone diameters recorded. Blunting of the zone of inhibition around the clindamycin disc indicates positive induction.

### 5.2.9 Transfer of plasmid resistance

#### 5.2.9.1 Mixed culture transfer (MCT)

In selecting strains for MCT isolates were tested for bacteriophage susceptibility, against phages J, 85 and 95, following the method in section 2.2.3 (Table 5.7). Strains that were lysed by phage were used in MCT experiments with a recipient that had been lysogenised by the corresponding phage (Table 5.8). All phages and recipient strains were supplied by Professor W.B. Grubb, Curtin University of Technology, Perth, Western Australia.

Table 5.7 Bacteriophage used in MCT experiments indicating their titre and propagating strain

| Bacteriophage | Titre (pfu/ml) | Propagating strain |
|---|---|---|
| J | $1.2x10^{10}$ | WBG248 |
| 85 | $3.1x10^{12}$ | WBG286 |
| 95 | $1.4x10^{10}$ | WBG696 |

The selection plates for antibiotic resistance plasmid transfer were prepared using brain heart infusion agar (BHIA) containing fusidic acid (5μg/ml) and rifampicin (25μg/ml) and the antibiotic, for which resistance transfer is being selected, at the appropriate concentration.

Table 5.8 Phage free *S. aureus* NCTC 8325-4 recipient strains, chromosomally resistant to rifampicin and fusidic acid, lysogenised by phage.

| Recipient strain | Lysogenised by phage |
|---|---|
| WBG879 | 85 |
| WBG1876 | J |
| WBG822 | 95 |

### 5.2.9.2 Resistance plasmid transfer by MCT

To 5 ml of BHIB (Oxoid, UK) containing 0.01 M $CaCl_2$ 100µl each of overnight BHIB cultures of donor and recipient isolates were inoculated. Broth mixtures were incubated for 24 hours at 37°C. Following incubation, cultures were centrifuged at 2,000rpm for 5 minutes and excess broth removed. The pellet was reconstituted following the addition of 100µl of BHIA. Each selection plate, containing fusidic acid (5µl/ml), rifampicin (25µl/ml) and the antibiotic for which resistance transfer was being selected, was inoculated with 50µl of culture. Dilutions of $10^{-7}$, $10^{-8}$ and $10^{-9}$ were prepared from the remaining 50µl of culture. One hundred microlitres of each dilution was inoculated onto Isosensitest Agar (Iso) (Oxoid, Basingstoke, UK) for transfer frequency estimation. All plates were incubated for 48 hours at 37°C before recording results. Any growth on selection plates after 48 hours of incubation indicated that resistance transfer had occurred. Transcipient organisms were cultured for purity on selection plates prior to examining co-transfer of resistance by the agar disc diffusion method (BSAC 2001).

### 5.2.10 Conjugation

#### 5.2.10.1 Polyethyleneglycol (PEG) method[433]

To a 20 ml glass universal 2 ml each of overnight BHIB culture of donor and recipient strains were mixed and pelleted by centrifugation at 2,000rpm for five minutes and the supernatant discarded. The cell pellet was reconstituted in 500µl of BHIB containing 40% PEG (4000) (Sigma, UK) and incubated overnight at 37°C with shaking. Cells were again pelleted by centrifugation (as above) and resuspended in 1ml of BHIB. From this mixture 100µl was plated onto selection plates containing fusidic acid, rifampicin (concentrations as above) and the resistance marker being transferred. The plates were incubated for 48 hours at 37°C before examination for growth. Estimation of transfer frequency was performed following the method in section 5.2.9.2. Any colonies found on plates were subcultured onto selection plates for analysis of resistance gene transfer. Donor and recipient isolates were inoculated seperately in 2 ml of BHIB and processed as above. These cultures were incorporated as transfer negative controls.

#### 5.2.10.2 Broth transfer

To a 20 ml glass universal a mixture of overnight BHIB cultures of donor and recipient strains was prepared at a ratio of 1:3 (recipient:donor) and incubated for 6 hours at 37°C. Cells were pelleted by centrifugation at 3,000rpm for five minutes and supernatant discarded. The cell pellet was reconstituted in 100µl of BHIB. From this mixture, 50µl was plated onto selection plates containing fusidic acid, rifampicin (concentrations as above) and the resistance marker being transferred. Estimation of transfer frequency was performed following the method in section 5.2.9.2. Plates were

incubated for 48 hours at 37°C before examination for growth. Any colonies found on plates were subcultured onto selection plates for purity assessment.

### 5.2.11 Plasmid extraction

To a 10ml glass universal of BHIB 3-5 colonies of each test isolate was inoculated and incubated for 6-7 hours at 37°C. Cells were pelleted by centrifugation at 3,000rpm for 10 minutes and supernatant discarded. The cell pellet was resuspended by vortexing, added to a 1.5ml microtube and centrifuged at 10,000rpm for 10 minutes and supernatant discarded. The cell pellet was reconstituted in 500µl of NET buffer and centrifuge at 10,000rpm for 10 minutes and supernatant discarded. The cell pellet was reconstituted in 250µl of buffer P1 (Qiagen, UK), 8µl of lysostaphin and 20µl of lysozyme, mixed by vortexing and incubated for 30 minutes at 37°C. The lysate was cooled to 20-25°C and 250µl of buffer P2 added. The suspension was gently inverted 4-6 times and incubated at room temperature for 5 minutes. Following incubation 350µl of chilled buffer N3 was added and immediately inverted 4-6 times and incubated on ice for 15 minutes. The suspension was centrifuged at 12,000rpm for 10 minutes, the supernatant decanted into a fresh 1.5ml microtube and further centrifuged at 12,000rpm for 10 minutes. The supernatant was applied to a Qiaprep spin column (Qiagen, UK) and centrifuge at 12,000rpm for 1 minute and the flow through discarded. The spin column was washed with 0.5ml of PB buffer, centrifuged at 12,000rpm for 1 minute and the flow through discarded. A further centrifugation was performed at 12,000rpm for 1 minute to remove any residual wash buffer. Plasmid DNA was eluted by applying 70µl of EB buffer and incubating at room temperature for 1 minute followed by centrifugation at 12,000rpm for 1 minute. Plasmid DNA was stored at 4°C until required.

### 5.2.12 Endonuclease digestion of plasmid DNA

Endonuclease digestion of plasmid DNA was performed following the method in section 3.2.4

### 5.2.13 Loss of resistance/plasmids

To 5 ml of nutrient broth, 5 µl of an overnight nutrient broth culture was added and incubated overnight with shaking at 37°C and 43°C. For three days this procedure was repeated. On day four, 1µl of the culture was diluted in 5ml of nutrient broth and plated out for single colonies on Columbia blood agar. Individual colonies were selected and screened for loss of resistance by disc diffusion susceptibility method. Plasmid extraction was performed on all suseptible isolates (section 5.2.11)

### 5.2.14 *tetK* probe labeled with Digoxigenin-11-dUTP

The *tet*K probe, labeled with non-radioactive digoxigenin-11-dUTP (DIG), was produced using primers *tet*K1 and 2 and PCR cycling conditions in Table 5.1, and the PCR DIG probe synthesis kit (Roche Applied Science, Germany). Probe synthesis took place in a mixture containing 5µl 10x concentration PCR buffer with $MgCl_2$, 5µl PCR DIG labeling mix, 1µl of primers *tet*K1 and 2 (50pmol), 4µl of the amplification product of *tet*K PCR made up to 50µl with molecular grade water (Sigma, UK). The DIG labeled PCR product was purified using the QIAquick® PCR purification kit.

### 5.2.15 QIAquick® PCR purification protocol

To purify PCR products 500µl of buffer PBI was added to 100µl of PCR product mixed thoroughly, applied to a QIAquick spin column and centrifuged at 13,000rpm for 30 seconds. The flow-through was discarded and the spin column washed in 0.75 ml of buffer PE and centrifuged at 13,000rpm for 30 seconds. The flow-through was again discarded and the spin column centrifuged at 13,000rpm for a further 30 seconds. The QIAquick spin column was placed in a clean 1.5ml microcentrifuge tube. DNA was eluted by the addition of 50µl of buffer EB (10 mM Tris-HCl, pH 8.5) to the centre of the QIAquick membrane, incubated at room temperature for 1 minute, and then centrifuged at 13,000rpm for 1 minute. Purified DNA was stored at -20°C.

### 5.2.16 Southern blot hybridisation

#### 5.2.16.1 Buffers and reagents

**Buffer 1**

To 1l of distilled water 11.61g of malic acid and 8.76g of NaCl was added and adjusted to pH7 using 1N NaOH (Sigma, UK).

**Buffer 2**

To 100ml of buffer 1 5g of blocking agent (Roche, Germany) was added, dissolved at 50°C then autoclave sterilised at 121°C for 15 minutes.

**Hybridisation buffer**

To 100ml distilled water 25ml of 20x sodium citrate-sodium chloride (SSC), 0.2ml 10% sodium dodecyl sulphate (SDS) (Qbiogene, UK), 1g blocking reagent (Roche Applied Science, Germany), and 0.1g laurylsarcosine (Sigma, UK) was added, dissolved at 50°C and stored at -20°C.

**Blocking reagent stock (10%)**

Ten grams of blocking reagent (Roche Applied Science, Germany) was added to 100ml of maleic acid. The blocking reagent was dissolved at 60°C for 16-18 hours and stored at -20 °C.

**0.5M NaOH-1.5M NaCl**

To 1l of distilled water 20g NaOH and 87.66g NaCl (Sigma, UK) was added and stored at room temperature.

**0.5M tris-HCl-3M NaCl pH8**

To 1l of distilled water 60.5g Trizma base and 87.66g NaCl was added, adjusted to pH8 with 1N HCl (Sigma, UK) and autoclave sterilised at 121°C for 15 minutes.

**20x SSC**

To 1l of distilled water 176g Sodium citrate and 351g NaCl was added, adjusted to pH7 with 1N NaOH and autoclave sterilised at 121°C for 15 minutes.

**2x SSC-0.1% SDS**

To 1l of sterile distilled water 50ml of 20x SSC and 5ml 10% SDS (Qbiogene) was added and stored at room temperature.

**0.2x SSC-0.1% SDS**

To 1l of sterile distilled water 5ml of 20x SSC and 5ml 10% SDS (Qbiogene) was added and stored at room temperature.

### 5.2.16.2 Vacuum blotting

Plasmid agarose gels were photographed, rinsed in distilled water and excess gel trimmed. A piece of Hybond N+ nylon was cut slightly larger, 5mm all round, than the gel and placed on the vacuum blotter platform. A window was cut in a vacugene mask, smaller than the nylon, placed over the nylon and the gel added to completely cover the mask window. A vacuum of 40 mm Hg was drawn and 50ml of 0.2 M HCl poured over gel and left for 4 minutes. The gel was neutralized by the addition of 50ml of 0.5 M NaOH-1.5 M NaCl (pH8) for 3 minutes followed by 50ml of 0.5 Tris-HCl-3 M NaCl (pH8) for 4 minutes. The gel was then flooded with 50ml of 20x SSC for 2 hours to allow the transfer of DNA from gel to Hybond N+ nylon. The bottom left hand corner of the nylon was marked with a T for orientation and DNA fixed by baking at 80°C for 2 hours.

### 5.2.16.3 DNA-DNA hybridization

**Prehybridisation**

The Hybond N+ nylon membrane was placed, DNA face up, in a glass hybridization tube (Hybaid™, UK) containing 5 ml of hybridization buffer. The tube was placed in a hybridization oven and prehybridisation allowed to proceed at 42°C for 2 hours.

**Hybridisation**

The labeled DNA probe was denatured at 95°C for 10 minutes followed by 4°C for 10 minutes and diluted 1:10 in tissue grade water (Sigma, UK). A 1:100 dilution of probe in hybridization buffer was added to the hybridization tube and hybridization allowed to proceed at 60°C for 16-18 hours.

**Post-hybridisation washes**

The hybridization solution was removed and stored at -20°C for reuse. The nylon membrane was washed twice in 0.2x SSC-0.1% SDS for 15 minutes at room temperature, followed by a further two washes in 0.2x SSC-0.1% SDS for 15 minutes at 65°C.

**Chemiluminescence detection with CDP Star™**

All incubations for the detection procedure were carried out at room temperature. After post hybridization washes the membrane was added to a Petri dish, 10ml of a 1% block buffer, prepared in buffer 1, was applied and gently shaken for 30 minutes. The nylon membrane was washed twice in buffer 1 for 15 minutes. Substrate dye (one tablet containing 45 µl NBT and 33 µl x-phosphate dissolved in 10ml tissue grade water) was applied to the membrane and incubated in a dark room for reaction development. The chemiluminescent reaction was stopped by washing in water; the membrane was dried then photographed.

## 5.3 Results

### 5.3.1. Detection of tetracycline resistance genes

One hundred and thirty four *S. aureus* isolates were examined for the presence of the tetracycline resistance genes. These included all 81 tetracycline resistant animal isolates and 53 tetracycline resistant human isolates, selected based on their year of isolation and PFGE pattern. All isolates tested carried at least one of the tetracycline resistance genes, *tet*K, *tet*L and *tet*M (Table 5.9 and Table 5.10). The *tet*O gene was not detected in any of the strains tested.

Table 5.9 Distribution of *tet* genes in *S. aureus* from animals

| Animal species | | Geographic source (n) | *tet* gene combination | | | | |
|---|---|---|---|---|---|---|---|
| | | | K | K+M | L | L+M | M |
| Livestock | Chicken | NI (30) | 30 | | | | |
| | Cow | G (28) | 10 | 10 | 4 | 2 | 2 |
| | Milk | G (6), SWS (1) | 7 | | | | |
| | Pig | H (5) | 5 | | | | |
| | Turkey | SWS (2) | 2 | | | | |
| Companion | Horse | USA (6) | | | | | 6 |
| | Parrot | USA (1) | | | | | 1 |
| Wild | Pheasant | H (1) | | | 1 | | |
| | Phillips goose hawk | NI (1) | | | | | 1 |
| Total | | 81 | 54 | 10 | 5 | 2 | 10 |

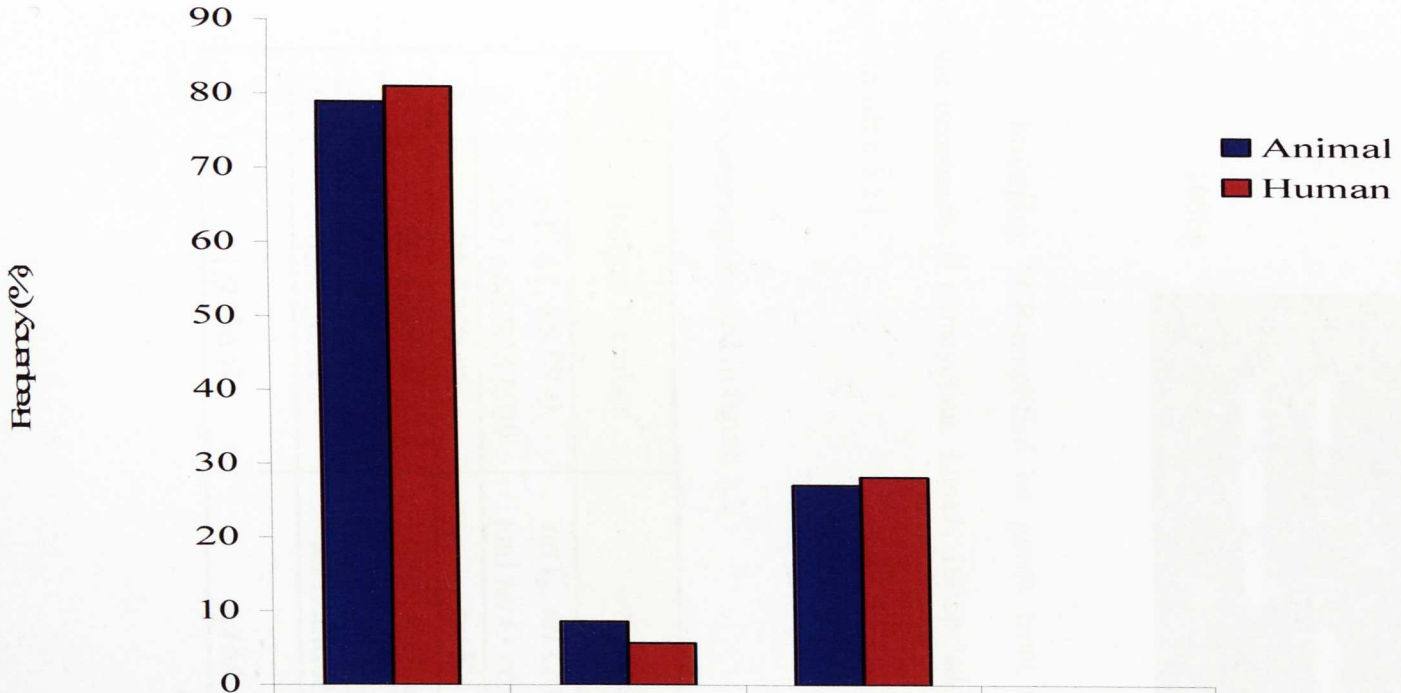
G, Grampian; H, Highland; NI, Northern Ireland; SWS, Southwest Scotland; USA, United States of America

Table 5.10 Distribution of *tet* genes in MRSA and MSSA in human

| PFGE type (n) | MSSA (n=20) *tet* gene combination | | | | MRSA (n=33) *tet* gene cobination | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | K | K+M | L+M | M | K | K+L+M | K+M | L+M | M |
| E15 (1) | | | | | 1 | | | | |
| E16 (6) | 2 | | | | 3 | | | | 1 |
| NARES (8) | 4 | | | 1 | 1 | | 1 | | 1 |
| 105 (4) | 1 | 1 | | | 2 | | | | |
| 105/112 (1) | | | | | | | | | 1 |
| 107 (2) | | | 1 | | | | | 1 | |
| 108 (2) | | | | | 1 | | | | 1 |
| 109 (2) | | | | | | | 1 | | 1 |
| 111 (2) | 1 | | | | 1 | | | | |
| 112 (1) | | | | | 1 | | | | |
| 116 (3) | | | | | 3 | | | | |
| 117 (1) | | | | | 1 | | | | |
| 118 (1) | | | | | 1 | | | | |
| 120 (1) | | | | | | 1 | | | |
| 121 (1) | 1 | | | | | | | | |
| 122 (1) | | | | | | | | | 1 |
| 124 (2) | 1 | | | | 1 | | | | |
| 126 (2) | 1 | | | | 1 | | | | |
| 127 (5) | 5 | | | | | | | | |
| 136 (1) | | | | | 1 | | | | |
| 141 (1) | | | | | | | | | 1 |
| 147 (1) | | | | | 1 | | | | |
| 153 (1) | | | | | 1 | | | | |
| 154 (1) | | | | | 1 | | | | |
| 155 (2) | 1 | | | | | | 1 | | |
| Total | 17 | 1 | 1 | 1 | 21 | 1 | 3 | 1 | 7 |

*tet*K was carried by 79% (64/81) of the animal isolates and 81.1% (43/53) of the human isolates and was the most frequent type of resistance gene observed (Figure 5.1). A total 54/64 (84.4%) animal isolates and 38/43 (88.4%) human isolates carried *tet*K alone. The remaining 10 (15.6%) animal isolates carried *tet*K in combination with *tet*M. These isolates were all from cattle from Grampian region. Five of the human isolates carried *tet*K in combination with *tet*M (4 isolates) and *tet*L + *tet*M (1 isolate). Twenty two (27.2%) animal isolates and 15 human isolates carried the *tet*M gene: 10 animal and eight human isolates carried it alone. Two animal and two human isolates carried *tet*M in combination with *tet*L. *tet*L was detected in seven animal and three human isolates. Only animal isolates carried *tet*L alone (Figure 5.2). The combination of *tet*K + *tet*L did not appear in any of the isolates tested. Among the human isolates a higher frequency of *tet*K was found among the MSSA (90%) while the frequency of *tet*M was greatest in isolates of MRSA (36.4%) (Figure 5.3). These differences were not statistically significant (Fisher's exact test $P= 0.96$ and $P= 0.37$ respectively).

Figure 5.1 Distribution of *tet* genes among animal and human *S. aureus*



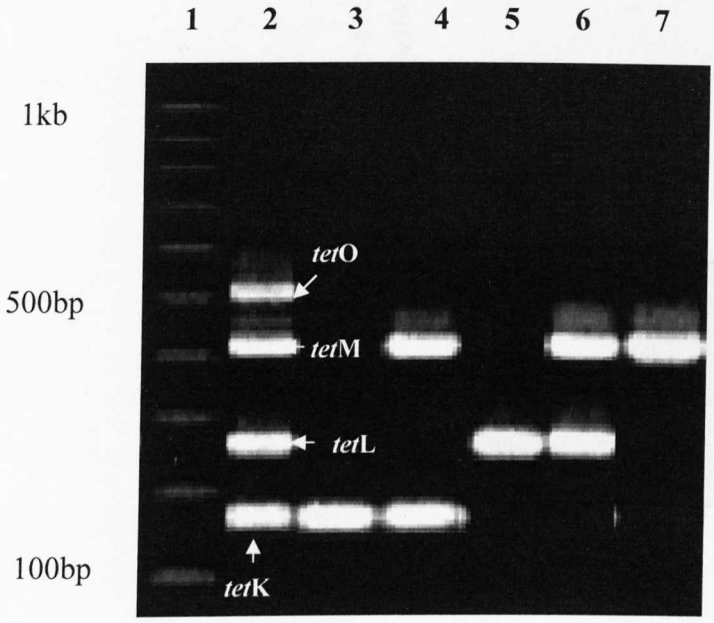
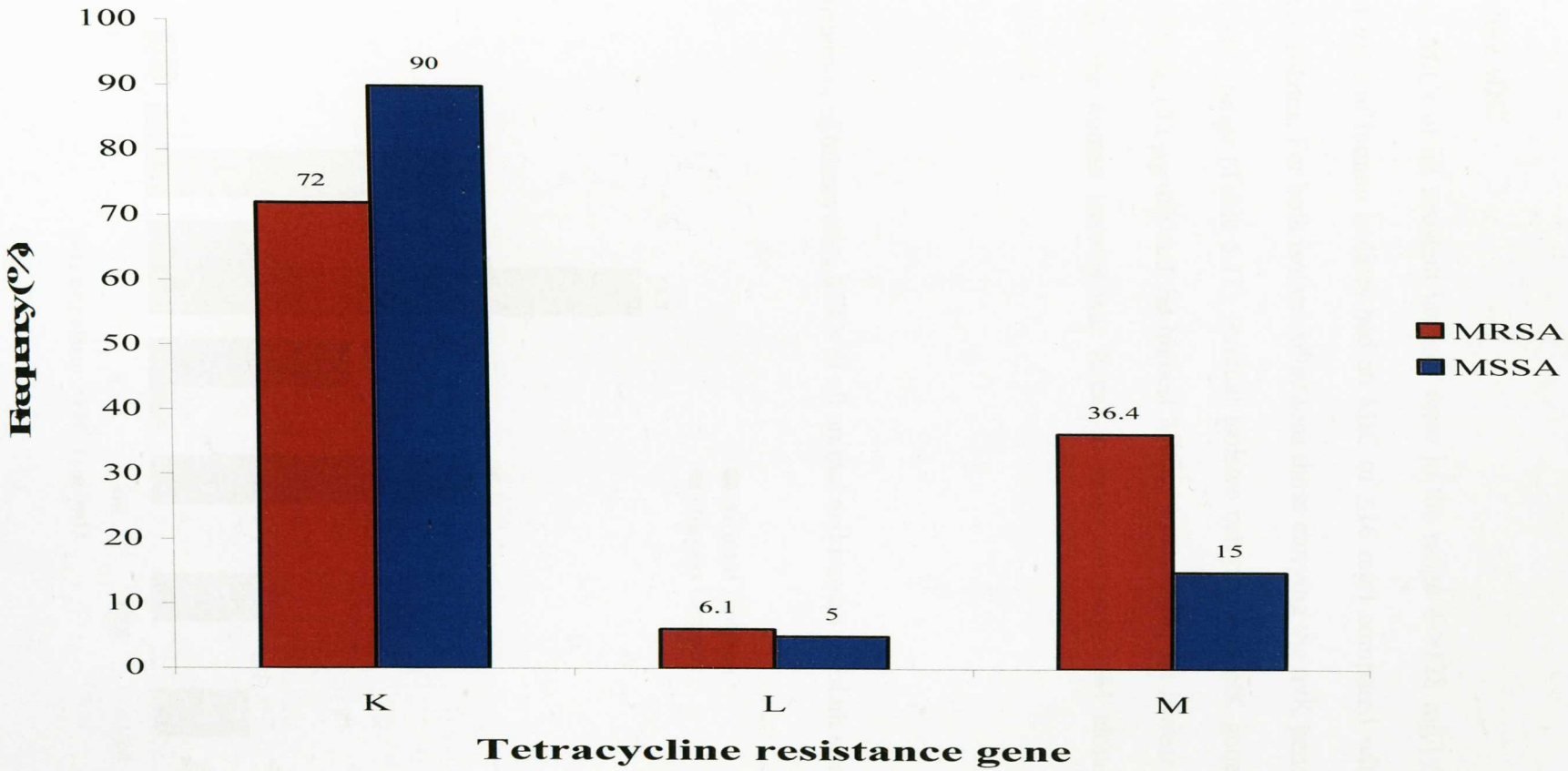



Figure 5.2 Multiplex PCR-amplified *tet* genes from representatives of *S. aureus* exhibiting resistance to tetracycline. Lane1, 100bp ladder; lanes, 2-7 correspond to isolates in table 5.11

Table 5.11 Isolates represented in figure 5.2

| Lane | Isolate Number | *tet* gene | Animal source |
|---|---|---|---|
| 2 | pT181, pSTS-9, 2567 and BM2509 | *tet* K, *tet* L, *tet* M and *tet* O controls | |
| 3 | 01.9740.M | *tet* K | Turkey |
| 4 | 02.4259.W | *tet* K and *tet* M | Cow |
| 5 | 01.9763.L | *tet* L | Pheasant |
| 6 | 02.4280.N | *tet* L and *tet* M | Cow |
| 7 | 01.9796.B | *tet* M | Phillips goose hawk |

Figure 5.3 Frequency of tetracycline resistance genes in MRSA and MSSA in humans



### 5.3.2 Tetracycline MIC

The tetracycline MICs of all resistant isolates were in the range 4->128 mg/l (Figure 5.4). A total of 98% of human isolates had an MIC of ≤16 mg/l compared with only 38.3% of animal isolates. For both isolate collections those carrying the *tet*K gene alone had the widest MIC range (Table 5.12). Animal isolates carrying the *tet*K gene alone had the lowest $MIC_{50}$ (32 μg/ml) and the highest $MIC_{90}$. In contrast, the lowest $MIC_{50}$ (8 μg/ml) among the human isolates was found in those carrying *tet*M alone or in combination with *tet*L.

Figure 5.4 Distribution of tetracycline MICs of all animal and human resistant strains

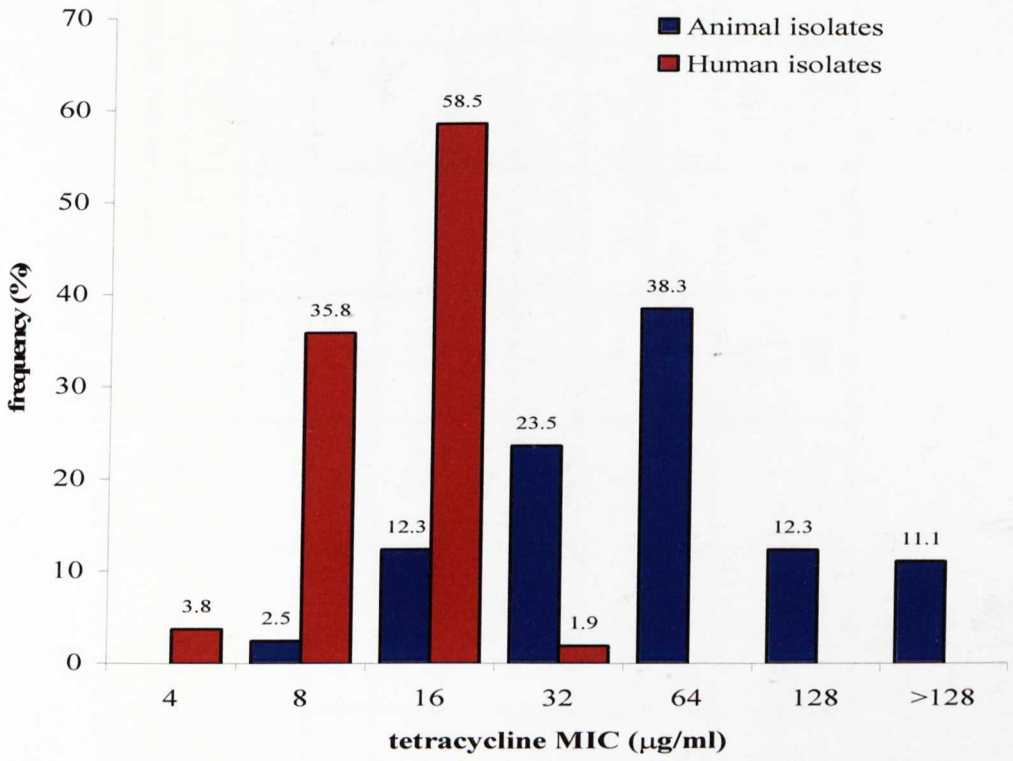


Table 5.12 Distribution of tetracycline MIC and *tet* genes in *Staphylococcus aureus* in animals and humans

| MIC (μg/ml) | No. isolates | *tet* gene combinations of animal isolates | | | | | No. isolates | *tet* gene combinations of human isolates | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | K | K+M | L | L+M | M | | K | K+L+M | K+M | L+M | M |
| 4 | | | | | | | 2 | 2 | | | | |
| 8 | 2 | 2 | | | | | 19 | 12 | | 1 | 1 | 5 |
| 16 | 10 | 10 | | | | | 31 | 23 | 1 | 3 | 1 | 3 |
| 32 | 19 | 16 | 2 | 1 | | | 1 | 1 | | | | |
| 64 | 31 | 14 | 4 | 3 | | 10 | | | | | | |
| 128 | 10 | 3 | 4 | 1 | 2 | | | | | | | |
| >128 | 9 | 9 | | | | | | | | | | |
| $MIC_{50}$ | 64 | 32 | 64 | 64 | 128 | 64 | 16 | 16 | 16 | 16 | 8 | 8 |
| $MIC_{90}$ | >128 | >128 | 128 | 128 | 128 | 64 | 16 | 16 | 16 | 16 | 16 | 16 |

### 5.3.3 *tet*K long PCR RFLP

Thirty representative *tet*K positive isolates, 15 animal and 15 human, were selected based on the animal source, and PFGE pattern. The *tet*K long PCR product for each isolate was examined (Figure 5.5) by restriction endonuclease digest, using the *Alu*I, *Rsa*I, *Hinc*III and *Kpn*I, and produced indistinguishable restriction patterns in line with computer predictions (Figures 5.6, 5.7, 5.8 and 5.9). The presence of identical resistance genes in *S. aureus* from different animal and human sources and from different clonal background suggests that the transfer of *tet*K between animal and human *S. aureus* isolates can and does occur.

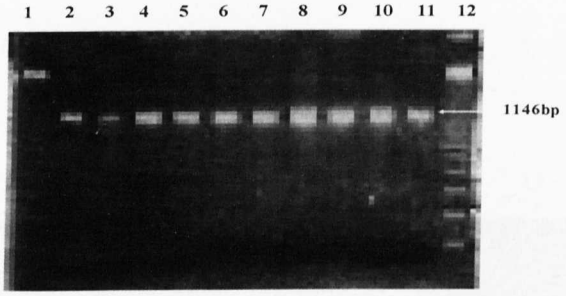



Figure 5.5 Undigested products of *tet*K long PCR. Lanes 1 and 12, 123bp molecular weight marker; lanes 2-11, correspond to isolates in table 5.13

Table 5.13 Isolates represented in figure 5.5

| Lane | Isolate No | PF type | Isolate source |
|---|---|---|---|
| 2 | 01.9709.W | A | Animal |
| 3 | 02.4276.N | T | |
| 4 | 02.4222.Q | D | |
| 5 | 01.9766.E | T | |
| 6 | 01.9743.D | A | |
| 7 | 02.5389.H | 16 | Human |
| 8 | 03.3797.B | 15 | |
| 9 | 03.1059.P | 105 | |
| 10 | 03.4741.G | 127 | |
| 11 | 03.3736.T | 111 | |

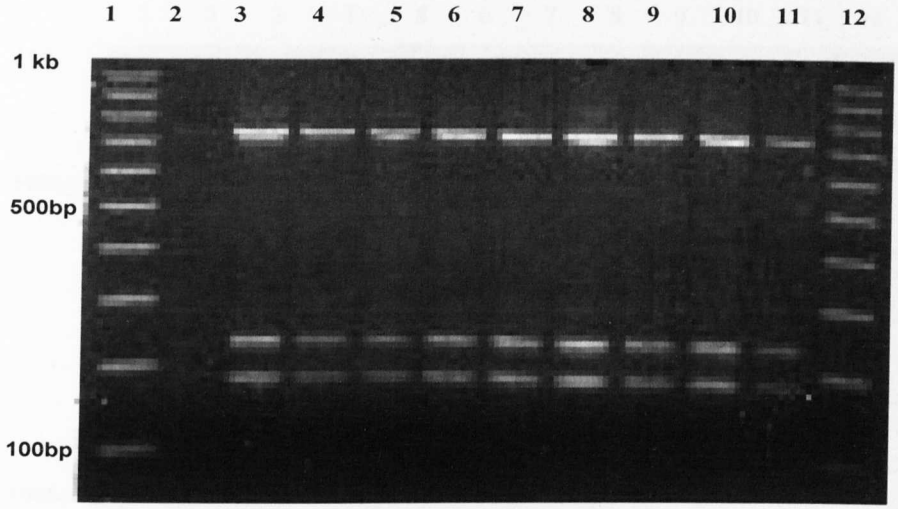



Figure 5.6 *Rsa*I restriction fragment digest patterns of *tet*K long PCR products. Lanes 1 and 12, 100bp molecular weight marker; lane 2, Water control; lanes 3-11, correspond to isolates in table 5.14

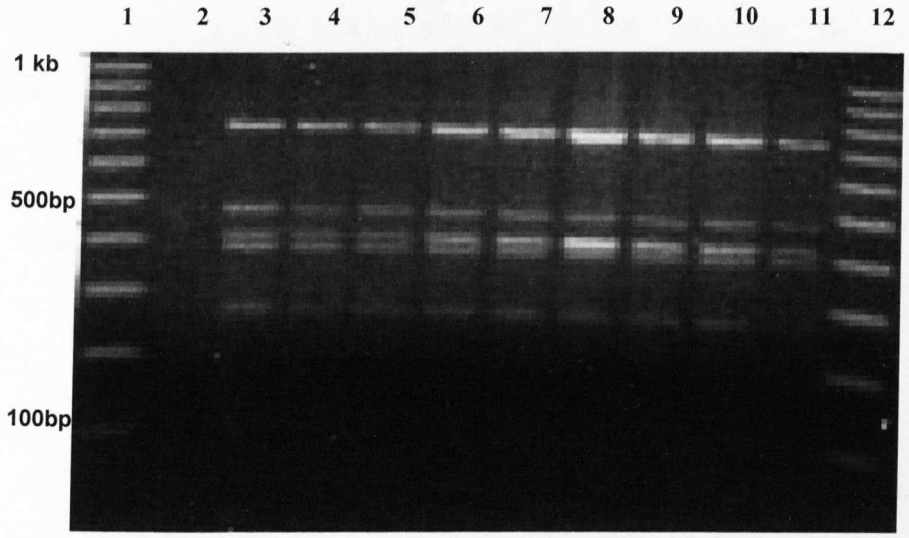



Figure 5.7 *Kpn*I restriction fragment digest patterns of *tet*K long PCR products. Lanes 1 and 12, 100bp molecular weight marker; lane 2, Water control; lanes 3-11, correspond to isolates in table 5.14

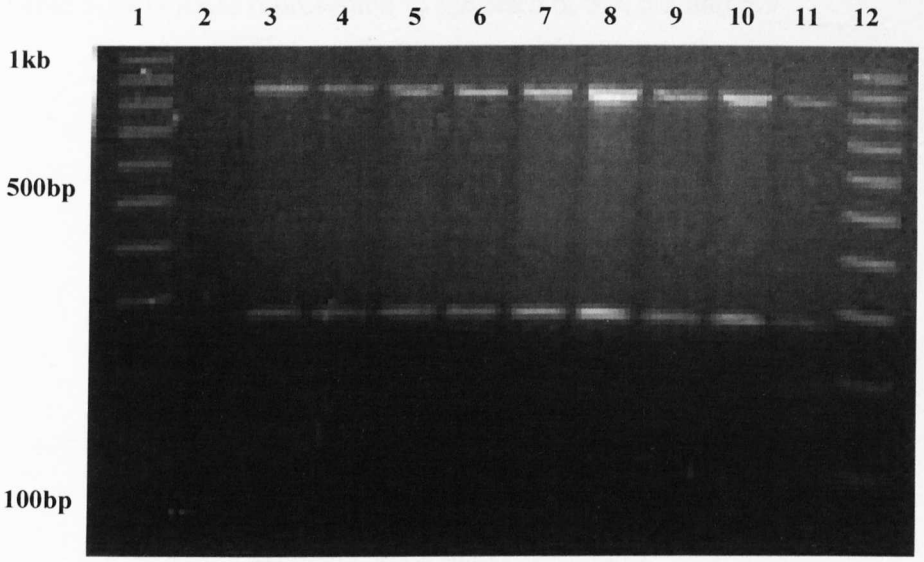



Figure 5.8 *Hinc*II restriction fragment digest patterns of *tet*K long PCR products. Lanes 1 and 12, 100bp molecular weight marker; lane 2, Water control; lanes 3-11 correspond to isolates in table 5.14

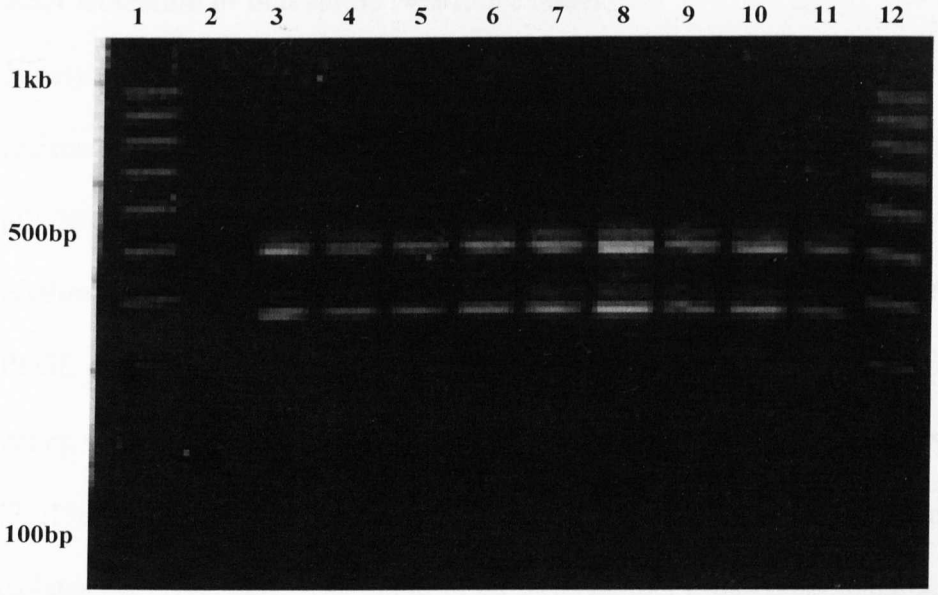



Figure 5.9 *Alu*I restriction fragment digest patterns of *tet*K long PCR products. Lanes 1 and 12, 100bp molecular weight marker; lane 2, Water control; lanes 3-11, correspond to isolates in table 5.14

Table 5.14 Isolates represented in figures 5.6, 5.7, 5.8 and 5.9

| Lane | Isolate No | PFGE type | Isolate source |
|---|---|---|---|
| 3 | 02.4276.N | T | Animal |
| 4 | 02.4222.Q | D | |
| 5 | 01.9766.E | T | |
| 6 | 01.9743.D | A | |
| 7 | 02.5389.H | 16 | Human |
| 8 | 03.3797.B | 15 | |
| 9 | 03.1059.P | 105 | |
| 10 | 03.4741.G | 127 | |
| 11 | 03.3736.T | 111 | |

### 5.3.4 Detection of macrolide resistance genes

Ninety five *S. aureus* isolates were examined for the presence of the erythromycin resistance genes *ermA*, *ermB* and *ermC* (Figure 5.10 and Figure 5.11). These included all 28 erythromycin resistant animal isolates (Table 5.1 and Table 5.2) and 67 erythromycin resistant human isolates, selected based on their year of isolation and PFGE pattern (Table 5.4). A total of 84.5% carried one of the three macrolide resistance genes. The *ermC* gene was the most frequently detected resistance gene in the animal (71.1%) and human (41.8%) isolate collections (Figure 5.12). Among the animal isolates the *ermC* gene was found in all three animal groups (companion, livestock and wild). A total of 7/28 (25%) of animal isolates and 20/67 (30%) of human isolates carried *ermA*. Only two isolates, one animal and one human, carried *ermB*. Eighteen isolates, all human, did not produce a PCR product using the primers listed in Table 5.5 and were classified as non-typable[384].

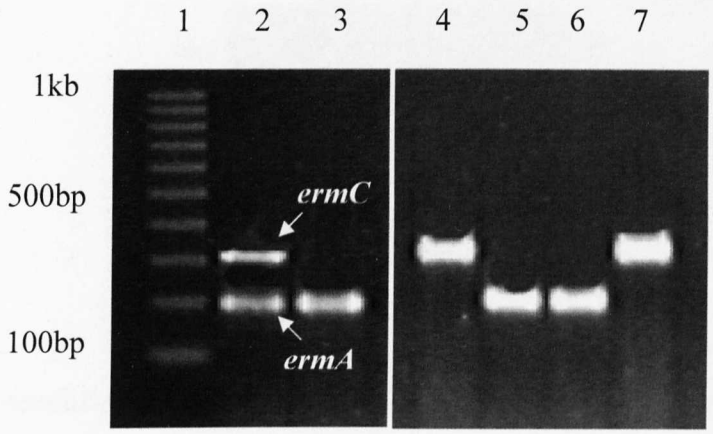



Figure 5.10 Multiplex PCR-amplified *ermA* and *ermC* genes from representatives of *S. aureus* exhibiting resistance to erythromycin. Lane 1, 100bp molecular weight ladder; lanes 2-7, correspond to isolates in table 5.15

Table 5.15 Isolates represented in figure 5.10

| Lane No | Isolate No | *erm* gene | Animal source |
|---|---|---|---|
| 2 | 2387/00 and 113ero-2222 | *ermA* and *ermC* | Control strains |
| 3 | 01.9796.B | *ermA* | Phillips goose hawk |
| 4 | 01.9760.J | *ermC* | Pheasant |
| 5 | 1408-1 | *ermA* | Dog |
| 6 | 1866 | *ermA* | Dog |
| 7 | 02.4265.C | *ermC* | Cow |

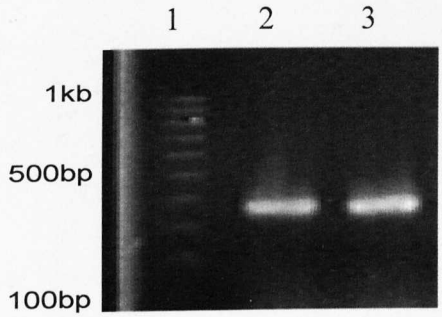
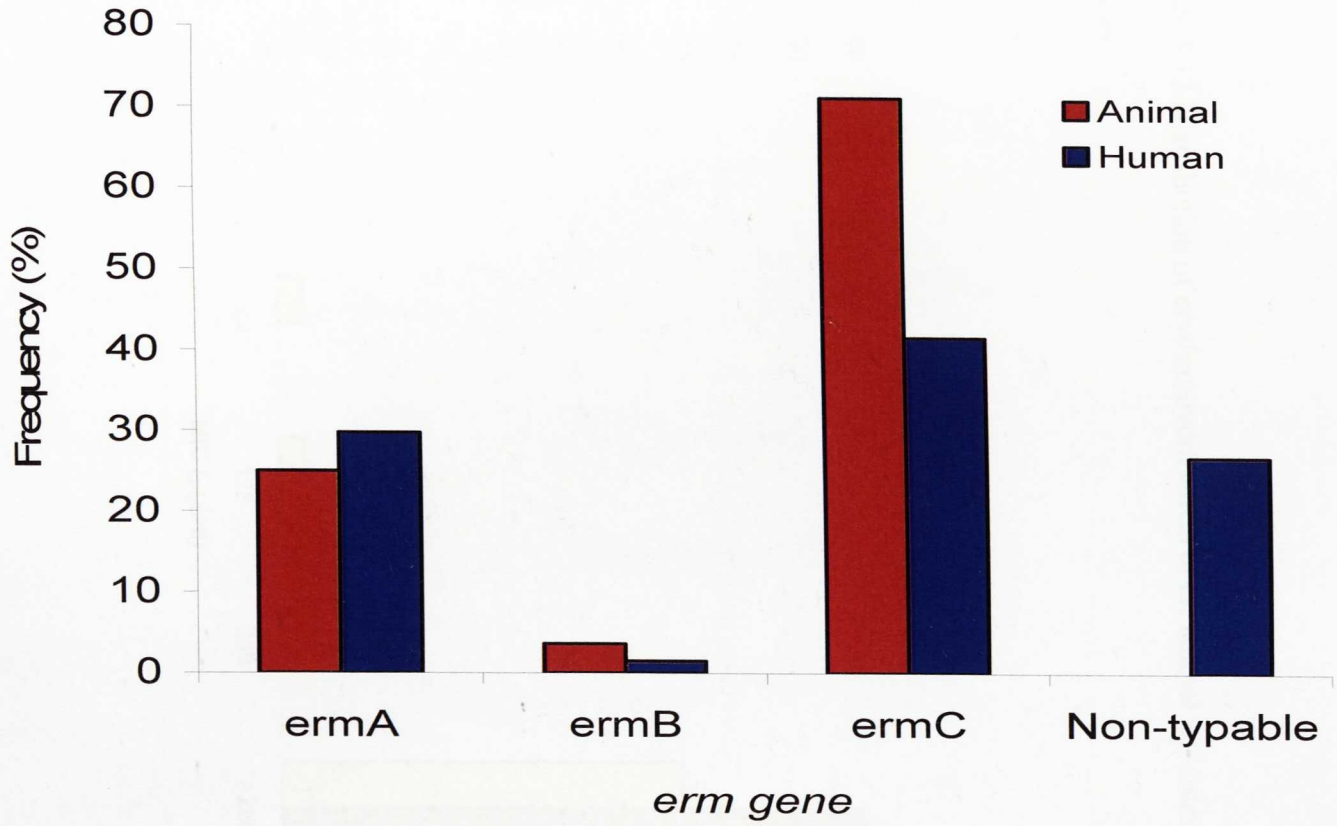



Figure 5.11 PCR-amplified *ermB* gene from representatives of *S. aureus* exhibiting resistance to erythromycin. Lane 1, 100bp molecular weight ladder; lane 2, RN11 *ermB* control isolate; lane 3, animal strain number 01.9763.L.

Figure 5.12 The frequency of *ermA*, *ermB* and *ermC* among animal and human *S. aureus* strains



### 5.3.5 Distribution of erythromycin MICs

The erythromycin MICs of all resistant isolates were in the range 32->256 mg/l with 31.4% of human isolates in the range 32-256 mg/l (Figure 5.13). All animal isolates had an MIC of >256 mg/l. Non-typable human isolates had the widest MIC range. However, the $MIC_{50}$ and $MIC_{90}$ for all gene classes was >256 mg/l (Table 5.16)

Figure 5.13 Distribution of erythromycin MICs of all animal and human resistant isolates

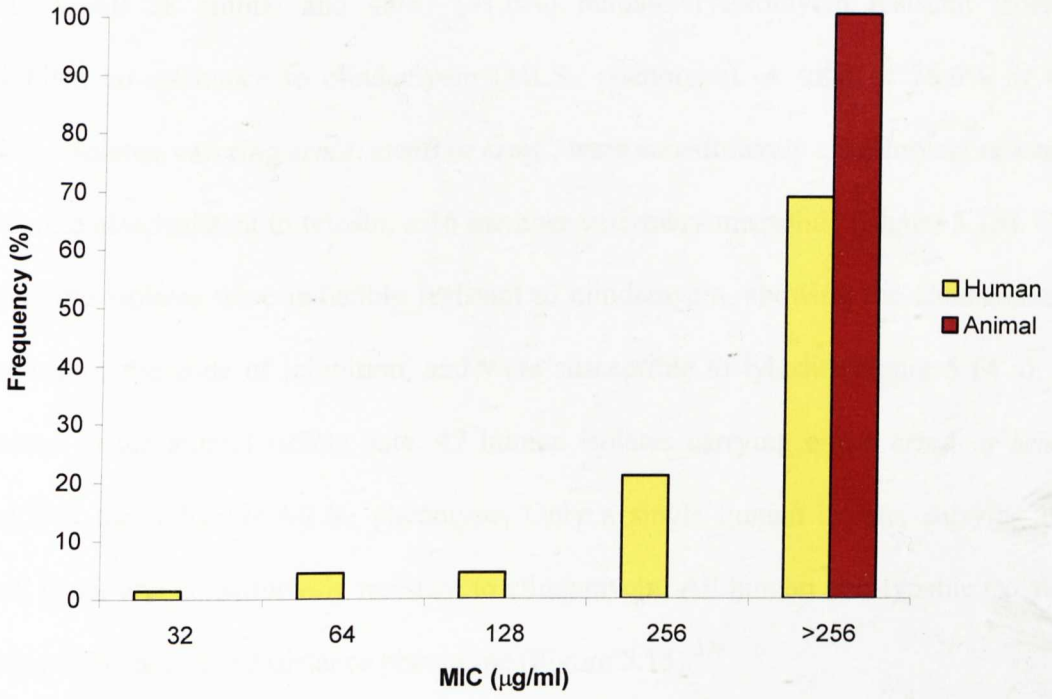


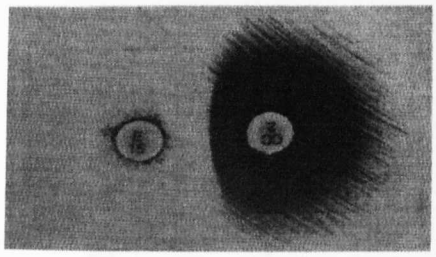
Table 5.16 Distribution of erythromycin MIC and *erm* gene in MRSA from humans

| Gene | Erythromycin MIC (mg/l) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 32 | 64 | 128 | 256 | >256 | $MIC_{50}$ | $MIC_{90}$ |
| *ermA* | | 1 | 1 | 5 | 13 | >256 | >256 |
| *ermB* | | | | | 1 | >256 | >256 |
| *erm* C | | 1 | | 5 | 22 | >256 | >256 |
| NT | 1 | 1 | 2 | 4 | 10 | >256 | >256 |

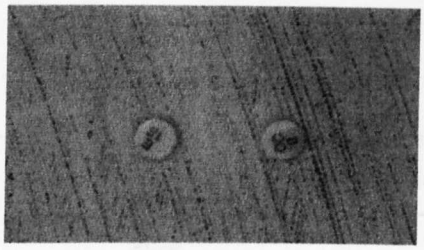
### 5.3.6 Induction of clindamycin resistance in erythromycin resistant *S. aureus* strains

Disc diffusion testing produced three distinct resistance phenotypes (Figure 5.14 a, b and c). All 28 animal and 48/67 (71.6%) human erythromycin resistant isolates exhibited co-resistance to clindamycin ($MLS_B$ phenotype). A total of 78.6% of the animal isolates, carrying *ermA*, *ermB* or *ermC*, were constitutively clindamycin resistant and were also resistant to tylosin, a 16 member veterinary macrolide (Figure 5.15). The remaining isolates were inducibly resistant to clindamycin, showing the characteristic blunting of the zone of inhibition, and were susceptible to tylosin (Figure 5.14 a). In contrast to the animal isolate data, 47 human isolates carrying either *ermA* or *ermC* exhibited the inducible $MLS_B$ phenotype. Only a single human isolate, carrying the *ermB* gene, was constitutively resistant to clindamycin. All human non-typable isolates exhibited the negative resistance phenotype (Figure 5.15).[378]

a



b



c

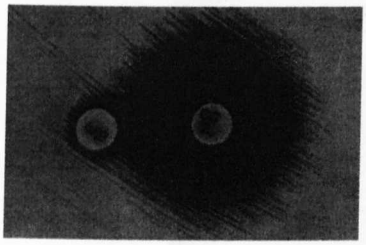
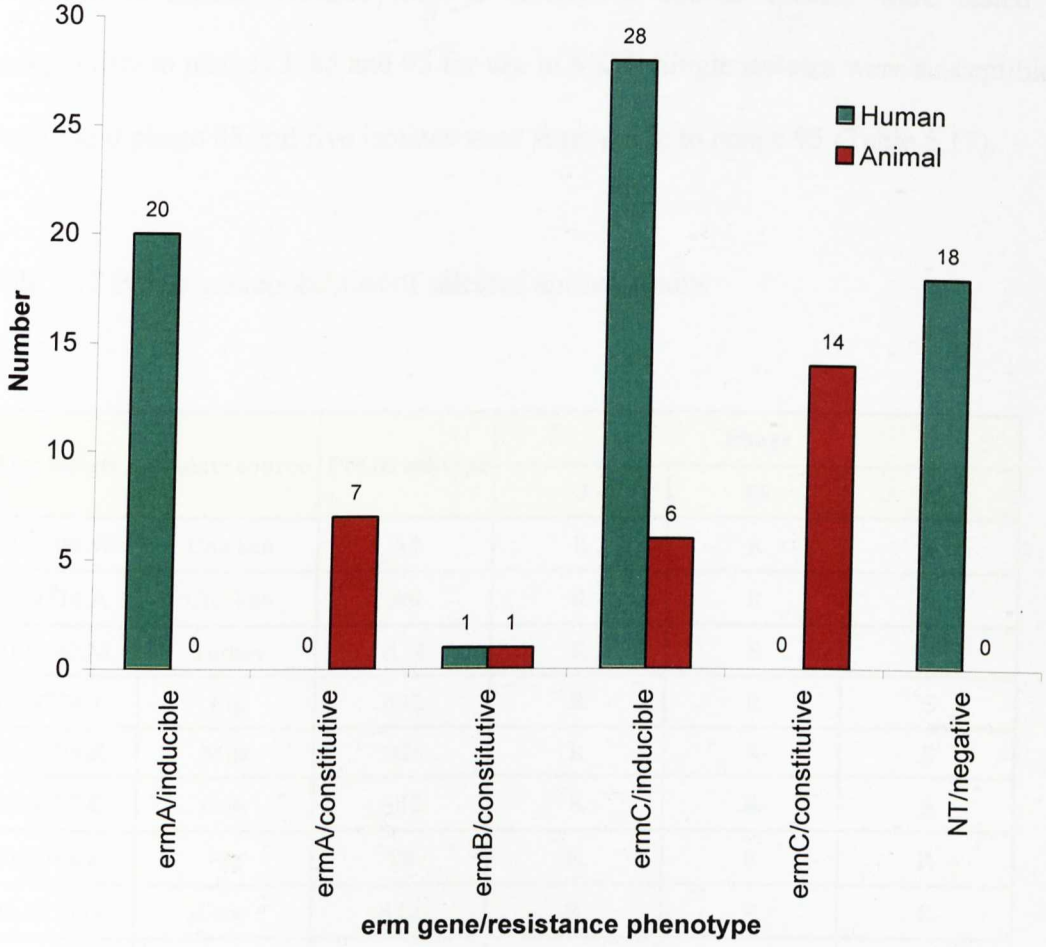


Figure 5.14 Examples of resistance phenotypes observed in double disc diffusion testing.Top row (A) Inducible phenotype, (B) Constitutive phenotype. Bottom row, (C) Negative phenotype.

Figure 5.15 Characteristics of animal and human erythromycin resistant strains




### 5.3.7 *tet*K resistance gene transfer studies

#### 5.3.7.1 Conjugation

Two methods of conjugation were used; the polyethylene glycol (PEG) and broth transfer methods. Seventeen randomly selected tetracycline resistant animal isolates, harbouring the *tet*K gene, were used as donors and WBG879, WBG1876 and WBG822 were recipient isolates. No resistance transfer was observed following either method of conjugation.

### 5.3.7.2 Mixed culture transfer (MCT)

Seventeen *S. aureus* isolates from a variety of animal species were tested for susceptibility to phages J, 85 and 95 for use in MCT. Single isolates were susceptible to phage J and phage 85 and five isolates were susceptible to phage 95 (Table 5.17).

Table 5.17 Phage susceptibilities of selected animal strains

| Test isolate | Isolate source | PFGE subtype | Phage | | |
|---|---|---|---|---|---|
| | | | J | 85 | 95 |
| 01.9709.W | Chicken | A7 | R | R | S |
| 01.9714.A | Chicken | A9 | R | R | R |
| 01.9740.M | Turkey | A14 | R | S | S |
| 01.9774.T | Pig | A12 | R | R | S |
| 01.2795.Z | Milk | D25 | R | R | S |
| 02.4277.E | Cow | A12 | R | R | S |
| 01.9768.P | Pig | T8 | R | R | R |
| 02.4275.G | Cow | A12 | R | R | R |
| 02.4218.Q | Milk | D15 | R | R | R |
| 02.4222.Q | Milk | D2 | R | R | R |
| 02.4263.W | Cow | T8 | S | R | R |
| 02.4257.Q | Cow | T3 | R | R | R |
| 02.4260.Z | Cow | T4 | R | R | R |
| 02.4259.W | Cow | T7 | R | R | R |
| 02.4288.R | Cow | T2 | R | R | R |
| 02.4276.N | Cow | T5 | R | R | R |
| 02.4256.Z | Cow | T4 | R | R | R |

Transfer of tetracycline resistance was detected only for isolate 01.9709.W and WBG822. The transfer frequency was $1.4 \times 10^{-4}$ transcipient/donor.

### 5.3.8 Verification of *tet*K gene transfer

The transcipient, donor and recipient isolates were tested for the presence of tetracycline resistance by broth microdilution MIC, *tet*K PCR, RS-PCR and PFGE. The transcipient had an MIC of 16 μg/ml, the donor MIC was 32 μg/ml, and the recipient was sensitive with an MIC of <0.06 μg/ml. By PCR the *tet*K gene was present in the transcipient and donor isolates and absent from the recipient strain (Figure 5.16). The transcipient and recipient strains produced RS-PCR and PFGE banding patterns that were indistinguishable and were dissimilar to those of the donor strain (Figures 5.17 and 5.18).

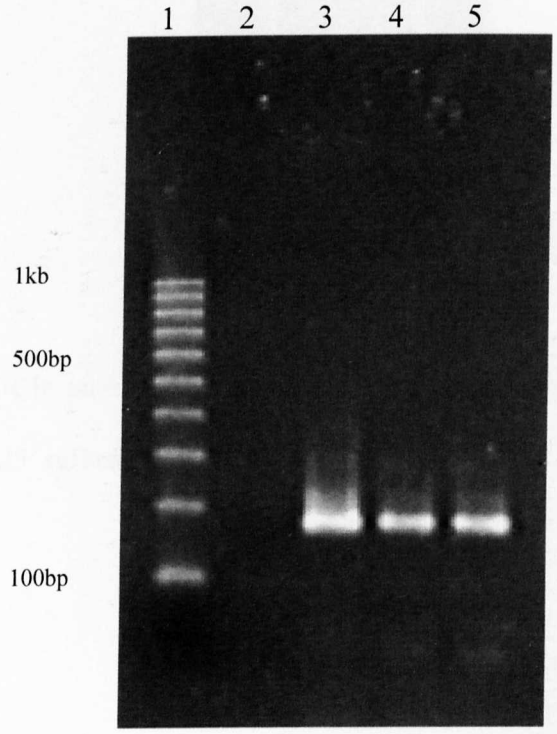



Figure 5.16 *tet*K PCR amplicons of donor, recipient and transcipient. Lane 1, 100bp molecular weight ladder; lane 2, tetracycline susceptible recipient; lane 3, pT181 positive control; lane 4, donor strain (WBG822) and lane 5, transcipient

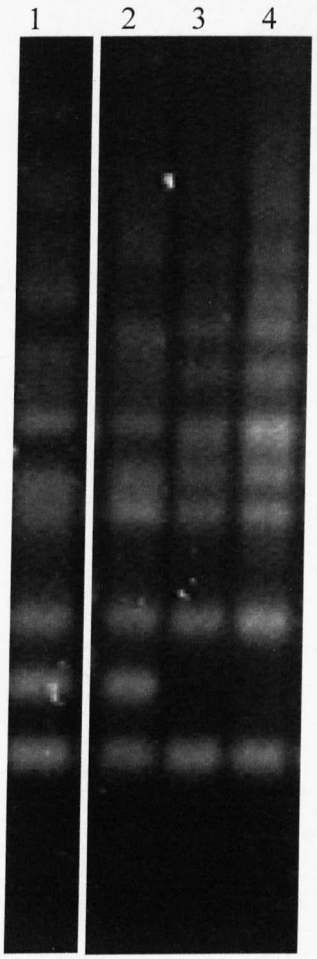



Figure 5.17 RS-PCR patterns of donor, recipient and transcipient strains. Lane 1. *S. aureus* NCTC8325 reference strain; lane 2, donor; Lane 3, recipient and Lane 4, transcipient

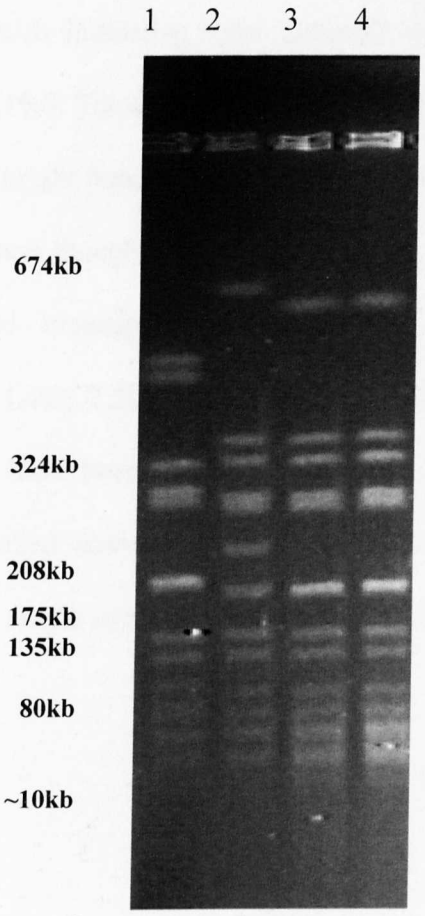
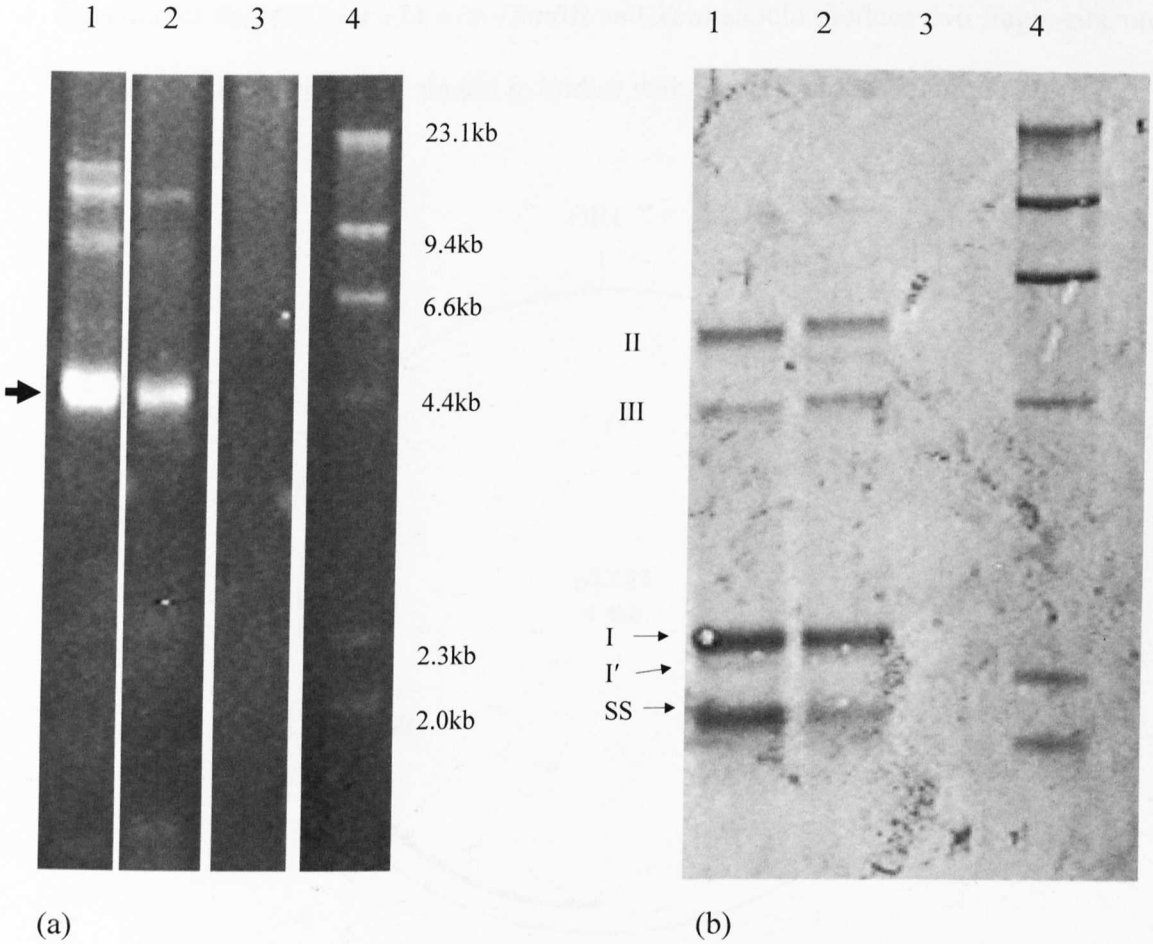



Figure 5.18 PFGE banding patterns of the donor, recipient and transcipient. Lane 1, donor; lane 2, *S. aureus* NCTC 8325 reference strain; Lane 3, recipient and Lane 4, transcipient

By plasmid extraction the donor strain contained four and the transcipient strain contained three plasmids indicating three plasmids were transferred during the MCT experiment (Figure 5.19a). The *tet*K gene has been found on plasmids ranging from 4.2-4.5kb. Therefore, the bright band, of approximately 4.4kb, indicated by the black arrow in lanes one and two was thought to be a *tet*K plasmid. Southern hybridisation revealed that both donor and transcipient contained four, and faint fifth, plasmids of approximately 5.5kb, 4.4kb, 2.3kb, 2.2kb and 2.0kb that hybridized with the *tet*K probe. These plasmid bands have been shown to represent; I, supercoiled plasmid DNA; I', covalently closed relaxed circular DNA; II, nicked open circular DNA; III, linear plasmid DNA and SS, single stranded plasmid DNA (Figure 5.19b).




(a) (b)

Figure 5.19 (a) Plasmid profiles of the donor, recipient and transcipient. Lane 1, donor; lane 2 transcipient; lane 3 recipient and lane 4 λ-DNA molecular weight marker. The bands marked with a black arrow indicate the predicted *tetK* plasmid. (b) Southern hybridization of donor, recipient and transcipient. Lane 1, donor; lane 2 transcipient; lane 3 recipient and lane 4 λ-DNA molecular weight marker. I, supercoiled plasmid DNA; I′, covalently closed relaxed circular DNA; II, nicked open circular DNA; III, linear plasmid DNA; SS, single stranded plasmid DNA.

An examination of the restriction enzyme recognition sites of plasmid pT181 that digestion of the *tet*K plasmid with *Hind*III and *Xba*I should produce two fragments, one of 2.3kb and one 4.4kb, that should hybridise with the *tet*K probe (Figure 5.20)[151].

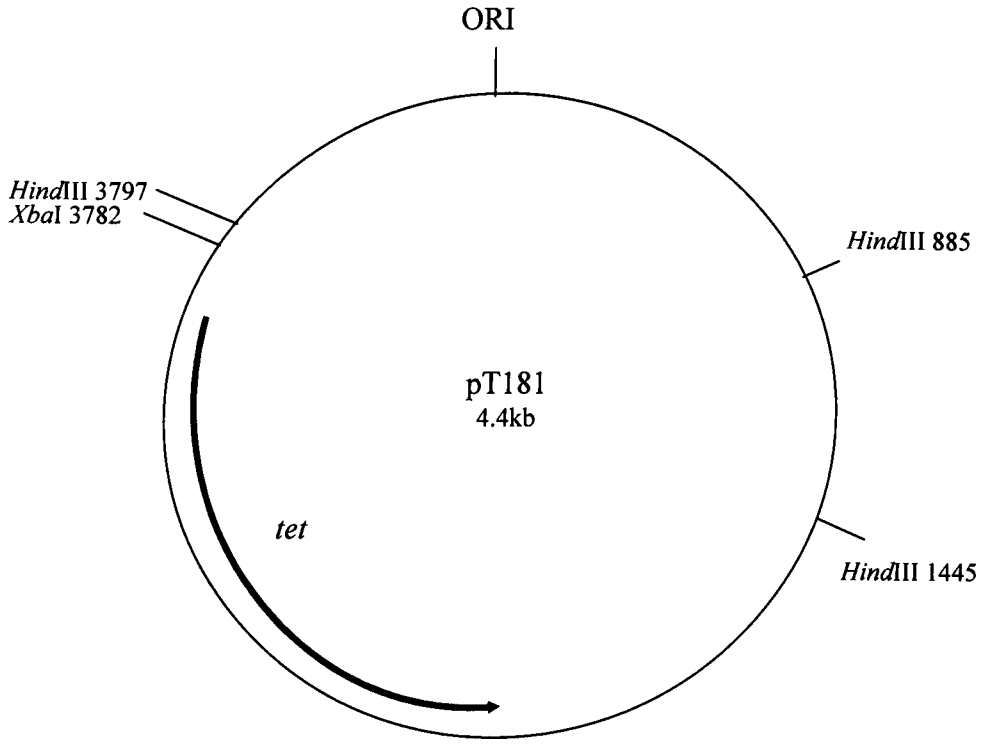



Figure 5.20 Restriction map of the tetracycline resistance plasmid pT181. ORI, origin of DNA leading strand synthesis; *tet*, tetracycline resistance gene.

Hybridisation of the plasmid endonuclease digests produced bands of the predicted size confirming the transfer of a 4.4kb *tet*K plasmid from 01.9709.W (a *S. aureus* isolated from a chicken) and WBG822 (*S. aureus* 8325-4 strain lysogenised by phage 95) (Figure 5.21).

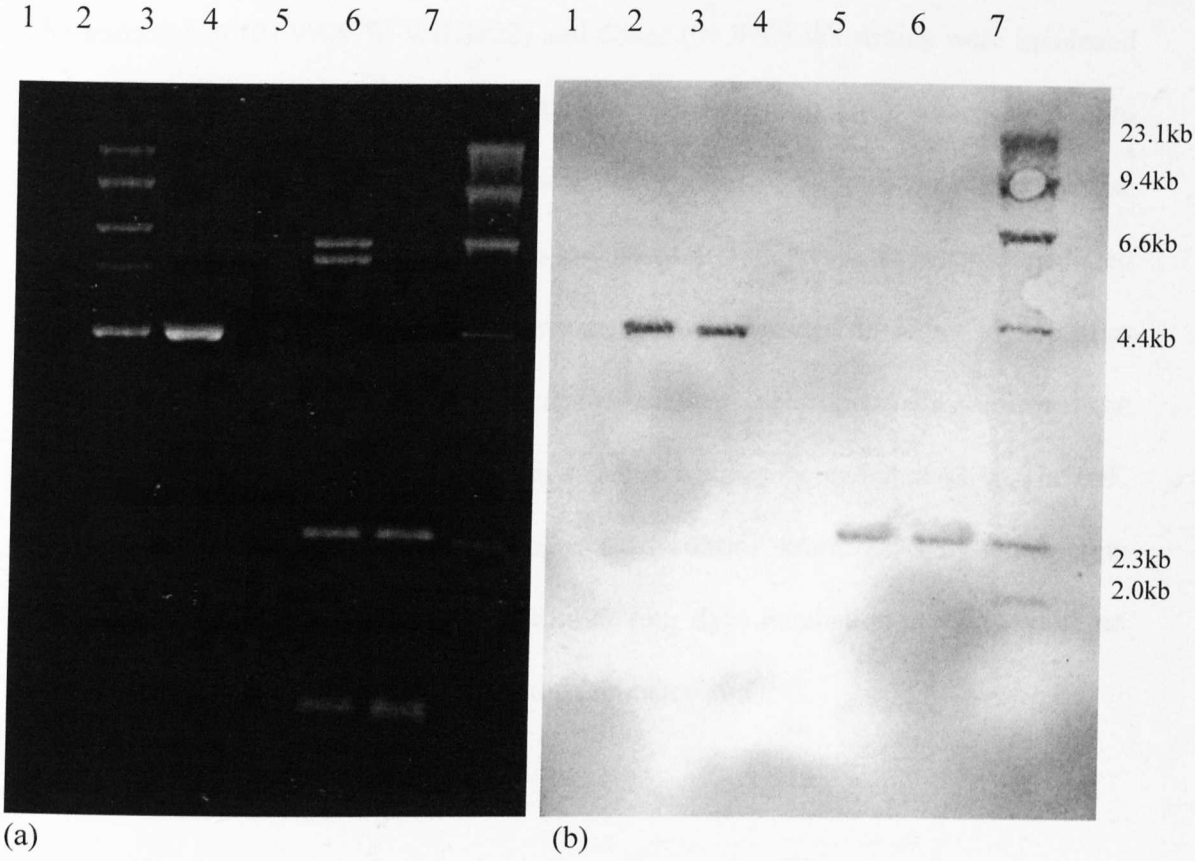



Figure 5.21 (a) *Hind*III and *Xba*I endonuclease digestion of plasmid extracts. Lanes 1-3, *Hind*III digest of WBG822 recipient, 01.9709.W donor and 01.9709.W-WBG822 transcipient; Lanes 4-6 *Xba*I digest of WBG822 recipient, 01.9709.W donor and 01.9709.W-WBG822 transcipient; Lane 7, λ-DNA DIG-labelled molecular weight marker. (b) Hybridisation of *Hind*III and *Xba*I endonuclease digests of *tetK* plasmid. Lanes 1-3, *Hind*III digest of WBG822 recipient, 01.9709.W donor and 01.9709.W-WBG822 transcipient; Lanes 4-6 *Xba*I digest of WBG822 recipient, 01.9709.W donor and 01.9709.W-WBG822 transcipient; Lane 7, λ-DNA DIG-labelled molecular weight marker.

### 5.3.9 Loss of resistance/plasmids

The transcipient (01.9709.W-WBG822) and donor (01.9709.W) strains were incubated for four days at 37°C and 43°C in nutrient broth (Oxoid, UK), subcultured onto Columbia blood agar for purity assessment then examined for resistance loss by disc diffusion. Donor and transcipient isolates incubated at 37°C retained plasmid mediated tetracycline resistance. No tetracycline resistance was detected in either isolate after incubation at 43°C. Plasmid extraction and subsequent electrophoresis confirmed the loss of all plasmids and of tetracycline resistance in isolates incubated at 43°C. The *tet*K positive control strain pT181 was included as a control strain. Loss of tetracycline resistance was also detected in this strain after four days incubation at 43°C while no loss of resistance was detected in the culture incubated at 37°C.

## 5.4 Discussion

### 5.4.1 Tetracycline resistance genes in *S. aureus* isolates

In the first part of this study the presence of the four specific tetracycline resistance genes (*tet*K, *tet*L, *tet*M and *tet*O), thus far found in staphylococci, was determined among animal and human *S. aureus* isolates. PCR characterization of the 134 tetracycline resistant strains examined showed they all carried one of the three tetracycline resistance genes, *tet*K, *tet*L and *tet*M alone or in combination and an absence of the *tet*O gene. Similar data on the prevalence of the tetracycline resistance genes *tet*K, *tet*L or *tet*M and absence of *tet*O among *S. aureus* isolate, between 1987 and 1998 from livestock, including cattle, pigs, rabbits, mink, turkey and duck, companion animals, including cat, dog, guinea pig and pigeon, and between 1997 and 1999 from humans, have previously been published by Schwarz *et al* and Bismuth *et al*[38,384].

The *tet*K gene is considered to be indigenous to staphylococci, often located on small plasmids, for example pT181, which can be mobilized or transduced between members of the same or related staphylococci[228]. Studies by Schmitz *et al*[374], Trzcinski *et al*[434], Bismuth *et al*[38], Ardic *et al*[23], Huys *et al*[196] and Aarestrup *et al*[5] on the presence of tetracycline resistance genes among *S. aureus* and CNS from animals and humans have all shown *tet*K to be the most prevalent tetracycline resistance determinant[38,384]. The *tet*K gene was also the most frequently detected *tet* gene among strains in this study, present in 79% of animal and 81.1% of human tetracycline resistant strains. Because of the omnipresence of the *tet*K gene among *S. aureus* isolates from animals, data from this part of the study indicate that *tet*K has established itself as an endemic tetracycline resistance determinant among animal *S. aureus* as it has in human *S. aureus*.

The *tet*L gene was originally found in various *Bacillus spp* but more recently it has been described in five other Gram-positive genera, including staphylococci, *Mycobacterium spp*, and *Streptomyces spp*, and in the Gram-negative anaerobes, *Fusobacterium spp*, and *Veillonella* spp[295,473]. Previous studies have shown a low prevalence of *tet*L in isolates of *S. aureus,* predominantly from pigs and in MRSA, but not MSSA, from humans[374,383,384]. Data from this study has also shown a low frequency of *tet*L (8.6%) in animal isolates, mainly from cattle but not pigs, and human isolates. Schwarz *et al* have also found the *tet*L gene to be present in 8% of tetracycline resistant staphylococci isolated from a selection of domestic and companion animals. In that study <1% of *S. aureus* carried *tet*L and it was the predominance of this gene among isolates of *S. sciuri* that accounted for the increased frequency[384]. In this study, no *tet*L was detected in *S. aureus* isolates from companion animals.

*tet*M was the second most frequently detected tetracycline resistance gene among animal and human *S. aureus* in this study. Mostly located on conjugative transposons, *tet*M genes have been shown to be easily transferred with these mobile elements[384]. Reports by Bismuth *et al*[38], Schmitz *et al*[374] and Ng *et al*[311] have also found *tet*M to be the second most prevalent *tet* gene in human MSSA isolates and the most prevalent gene in isolates of MRSA. While *tet*M can be found in combination with *tet*K, they all report that *tet*K is never found alone in human MRSA. In contrast, Trzcinski *et al* have shown *tet*K to be the sole source of tetracycline resistance in 32% of human MRSA isolates from Poland[434]. Seven isolates of MRSA, from companion animals, were examined in this study and all carried *tet*M as the sole source of tetracycline resistance. These animal isolates were previously characterized (data not included) as MRSA USA100, belonging to MLST ST5, a common human epidemic clone[229]. This suggests

a similar predilection of *tet*M for animal MRSA or it is also possible that this represents MRSA transmission between humans and companion animals[387]. The transmission of MRSA (EMRSA15) from humans to companion animals has previously been reported by Manian and Guardabassi *et al*[168,280]. A report by Voss *et al,* in 2005, found pig to human transmission of MRSA multi-locus sequence type ST398 in The Netherlands[461]. de Neeling *et al* and Khanna *et al* have documented a high prevalence of this MRSA clone in slaughter pigs in The Netherlands and Canada[95,229]. It is believed that this *S. aureus* lineage originated in pigs and has been the cause of serious human infections in The Netherlands, Germany and Austria[193,487]. In contrast, a recent report by Jones *et al* has shown that not only was *tet*K the most prevalent *tet* gene, among human MRSA and MSSA isolates, but was the sole source of tetracycline resistance in 73.9% and 73.8% of isolates respectively[171]. In this study there was a variation in the distribution of the *tet* genes between human isolates of MRSA and MSSA where the frequency of *tet*M among MRSA was double that detected in MSSA.

Among both isolate collections the combined presence of *tet*K + *tet*M and *tet*L + *tet*M was seen at relatively low frequencies. In neither isolate collection was the combination *tet*(K) + *tet*(L) seen. A single human MRSA isolate simultaneously carried *tet*(K), *tet*(L) and *tet*(M). Bismuth *et al*, in a study on gene heterogeneity for tetracycline resistance in staphylococcal species from humans, have reported the presence of *tet*K + *tet*L in *S. aureus* and *tet*K, *tet*L + *tet*M in *S. epidermidis*[38]. It has been reported by Schmitz *et al* that the combination of *tet*K + *tet*M genes was approximately 10 times more prevalent in tetracycline resistant MRSA than MSSA[375]. There was a difference in the distribution of this gene combination in isolate s from this study. However, the overall number of isolates carrying both *tet*K and *tet*M was low making a comparison difficult.

Tetracycline resistance caused by the *tet*O gene was first detected in campylobacter, enterococci and streptococcal species of human and animal origin and have a common ancestor with *tet*M[109,110,341,501]. Reports of the *tet*O gene in staphylococci are rare. The TetO determinant is not associated with conjugative elements which accounts for it being less widely distributed that *tet*M[73]. The *tet*O gene was not detected in either of the *S. aureus* isolate collections in this study. Although, Schwarz *et al* have reported the presence of *tet*O in the chromosomal DNA of *S. intermedius* and *S. xylosus* isolated from domestic animals[384].

The almost identical distribution of *tet* genes in animal and human *S. aureus* in this study shows that similar *tet* genes encode tetracycline resistance among *S. aureus* from these different reservoirs.

### 5.4.2 Analysis of *tet*K long PCR RFLP products

RFLP, of *tet*K long PCR products, was performed to determine the genetic relatedness of *tet*K genes from animal and human *S. aureus* isolates. Endonuclease digestion, with four restriction enzymes, of the *tet*K long PCR products of 15 animal and human tetracycline resistant isolates revealed these genes to be genetically homogeneous. No previous reports on the structural similarities of the *tet*K genes isolated from animal or human *S. aureus* could be founding a search of the online database of the United States national library of medicine (http://www.pubmed.gov). Data published by Salyers *et al*, on the sequence analysis of the *tet*Q gene found in different Gram-negative anaerobes has shown sequence similarities of 96-100%. Salyers *et al* concluded that this high sequence similarity supported the hypothesis that the gene had been spread by horizontal gene transfer and ruled out convergent evolution, the independent evolution

of two versions of the same gene[202]. As the *tet*K gene is located on small highly mobile plasmids it is likely that the presence of indistinguishable *tet*K genes in *S. aureus* isolated from animals and humans may be due to horizontal gene transfer between these two populations, although the direction of transfer remains unknown. It is also possible that the *tet*K genes in both animal and human strains may have been introduced from bacteria from different ecosystems. Therefore, both the source and direction of transmission of *tet*K remains to be confirmed.

### 5.4.3 Tetracycline MICs

The tetracycline MIC for all isolates was determined by the broth microdilution method. Results show a wider tetracycline MIC range for animal *S. aureus* strains (8->128mg/l) compared to that of the human strains (4-32mg/l) and of those published by Aarestrup *et al*[5,10] in *S. hyicus* from pigs (4-64mg/l) and *S. aureus* from poultry (4-64mg/l) from Denmark and Vancraeynest *et al* in *S. aureus* from rabbits (16-64mg/l) from five European countries[454]. The higher MICs in isolates from this study, compared to those of animal isolates from Denmark, are likely a reflection of the higher levels of tetracycline consumption in UK agriculture[3,428]. Over 98% of human isolates had an MIC of ≤16 mg/l while 85% of animal isolates had an MIC of ≥32 mg/l. The low MIC levels seen among human isolates in this study contrasts with data from Bismuth *et al*, Trzcinski *et al* and Sekiguchi *et al* who have all reported tetracycline MICs ranges of between 16-512mg/l among human *S. aureus* isolates from France, Poland, Turkey, Bulgaria, Latvia, Slovenia, Hungary, Russia and Japan[38,388,434]. Over the past decade, as the frequency of tetracycline resistance has increased, there has been a reduction in the use of tetracyclines in the treatment of human *S. aureus* infections which may account for the lower MICs compared to those seen in other countries[351].

In a review, of tetracycline resistance determinants in 1996, Roberts reported that an interesting property of ribosomal protection proteins is that they do not normally confer high level tetracycline resistance, as compared to the efflux genes[353]. Recent reports have shown tetracycline MICs for human *S. aureus* isolates carrying *tet*K or *tet*M alone to be comparable. Among the human isolates those carrying *tet*K or *tet*M alone had identical $MIC_{50}$ and $MIC_{90}$ results. Conversely and consistent with Roberts, animal isolates in this study carrying the *tet*K gene alone had a fourfold higher MIC than isolates carrying the *tet*M gene alone[353]. A low number of isolates, in both animal and human *S. aureus* populations, carried the *tet*M gene making a comparison between the two populations unfeasible. A study by Bismuth *et al* reported that the gene combination *tet*K + *tet*M had an additive effect on the tetracycline MIC compared to *tet*K or *tet*M alone[38]. In neither population in this study did the presence of multiple *tet* genes result in an appreciable increase in the tetracycline $MIC_{50}$ or $MIC_{90}$.

### 5.4.4 *tetK* resistance gene transfer

#### 5.4.4.1 Conjugative transfer

From plasmid profiling, it was observed that the *tet*K positive strain carried a plasmid with the estimated size of 4.2-4.4 kb which indicated the presence of a pT181-like plasmid[227]. The *tet*K-containing plasmid pT181 (GenBank accession number J01764) is regarded as the prototype of a family of small naturally occurring transmissible plasmids that are known to occur not only in clinical *S. aureus* but also in tetracycline resistant staphylococci from the environment, wild rodents and insectovars[177].

The ability of *S. aureus* strains isolated from animals to transfer plasmid mediated tetracycline resistance was studied in conjugation and mixed culture transfer experiments. Seventeen isolates from domestic animal species representing the major PF groups were selected. Of the seventeen isolates selected for transfer by PEG and broth mating no resistance gene transfer was detected. Resistance gene transfer among staphylococci by conjugation has previously been reported[438,440]. Plasmid mediated resistance was successfully transferred between laboratory strains of *S. aureus* and *S. epidermidis* by McDonnell *et al* and Forbes *et al* by filter mating at transfer frequencies of $10^{-6}$-$10^{-8}$ [141,288]. The transfer of mupirocin and chloramphenicol resistance was undertaken by Udo *et al* between laboratory isolates of coagulase negative staphylococci (CNS) and *S. aureus*[437]. They reported no plasmid mediated resistance transfer between CNS and *S. aureus* and suggest that this may be due to the presence of a restriction-modification barrier[440]. The role of restriction-modification systems is to prevent the uptake of potentially harmful or lethal DNA, such as bacteriophages, or prevent the acquisition of superfluous genes that may compromise fitness due to increased metabolic demand[463]. Waldron and Lindsay have shown substantial variations in the genes encoding SauI (*sau1hsdS* genes), a lineage specific type I restriction-modification system that blocks horizontal gene transfer in and between *S. aureus* isolates of different lineages. They also revealed that these differences corresponded to the major *S. aureus* lineages and suggest that horizontal gene transfer within lineages occurs at a higher frequency than between lineages[463]. The isolates used in this study belonged to the major MLST groups found among the animal collection. These included ST5, ST9 and CC97. The recipient strain used in this study was, *S. aureus* 8325-4, a human strain belonging to ST8. Therefore, conjugative transfer of tetracycline resistance between these strains may have been inhibited by the presence of lineage

specific restriction-modification systems. A previous study by Shoemaker *et al* has shown that conjugative tetracycline resistance gene transfer exhibited regulated transfer requiring the donor to be initially pre-treated with low levels of tetracycline. After induction the rate of transfer increased by 1000-10,000 fold[394]. Antibiotic pre-treatment of bacteria induces an SOS response that has been shown to promote the transfer of conjugative resistance elements. In contrast to the data from Shoemaker *et al*[394], Beaber *et al*[32] have shown that the SOS response could not be induced by antibiotics to which the donor strain is resistant. None of the 17 donor isolates used in this study was initially pre-treated with tetracycline. Therefore, it is possible that conjugative transfer of the *tet*K gene may have occurred but at such a low frequency that it could not be detected under these experimental conditions. A study by Udo *et al* was also unsuccessful in transferring plasmid mediated tetracycline resistance between *S. aureus* strains by conjugation[439].

### 5.4.4.2 Mixed culture transfer

Early experiments on the transfer of resistance plasmids in mixed broth cultures indicated that successful transfer was dependent on calcium ions and investigators attributed this method of transfer to be phage mediated conjugation[439]. Isolates used in mixed culture transfer were selected on the basis of phage susceptibility to phages J, 85 and 95. Four isolates exhibited susceptibility to phages 85 or 95 and were mated with the corresponding lysogenised *S. aureus* isolates. A single donor *S. aureus* isolate (01.9709.W) from a chicken, belonging to ST5, transfered *tet*K mediated resistance to the recipient isolate WBG822 (*S. aureus* 8325-4 lysogenised with phage 95) at a transfer frequency of $1.4 \times 10^{-4}$. Udo and Grubb have also shown phage mediated tetracycline resistance transfer between a human clinical *S. aureus* strain and a

laboratory mutant strain, although at a much lower transfer frequency than seen in this study[439]. Initially resistance transfer was established by broth microdilution MIC and *tet*K PCR of the transcipient strain together with donor and recipient. To confirm that plasmid transfer had occurred between 01.9709.W and WBG822, the donor, recipient and transcipient strains were analysed by RS-PCR, PFGE, plasmid isolation, plasmid restriction digest and subsequent Southern hybridisation. The transcipient was shown by RS-PCR and PFGE to have banding patterns identical to WBG822. Three plasmids of approximately 15 kb, 9 kb and 4.4 kb were transferred between donor and recipient. DNA-DNA hybridisation analysis revealed five bands demonstrating homology to the *tet*K probe in the plasmid extracts of donor and transcipient but not the recipient strain. Experiments on the in vitro replication of the *tet*K plasmid, pT181 by Khan *et al*, have shown that this plasmid could be present intracellularly in five different structural forms[227]. Southern blot analysis of plasmid pT181 revealed five bands hybridising with a *tet*K probe and was subsequently shown to correspond to; super coiled plasmid DNA; covalently closed relaxed circular DNA; nicked open circular DNA; linear plasmid DNA; and single stranded DNA. These bands were consistent to those seen in the initial hybridisation experiment. Restriction analysis, using *Hind*III and *Xba*I, and hybridisation analysis of the plasmid extracts of donor and recipient isolates confirmed the horizontal transfer of a 4.4 kb, pT181-like, plasmid.

Incubation of the donor and transcipient isolates for four days at 37°C and 43°C resulted in the stable maintainence of the 4.4 kb plasmid and in the expression of tetracycline resistance at 37°C and the loss plasmid mediated tetracycline resistance at 43°C.

These data verify that the horizontal transfer of a tetracycline resistance plasmid between strains 01.9709.W, isolated from a chicken, and WBG822 had occurred. The frequency of plasmid transfer in this study was also higher than previously reported by McDonnell *et al* and Forbes *et al*[141,288].

### 5.4.5 Macrolide resistance genes in *S. aureus* isolates

In *S. aureus* a number of genes confer resistance to macrolides predominantly by target site modification. The genes responsible for this target site modification have been designated *ermA*, *ermB*, and *ermC* and are present in 94-98% of resistant isolates[478]. Additionally, and at very low frequencies, resistance can also be caused by inactivation, encoded by *ereA* and *ereB*, and active efflux, encoded by *msrA* and *msrB* [263,354,384,474].

All of the 28 animal and 56/77 (73.1%) human isolates carried one of the *erm* genes. The *ermC* gene was the most prevalent macrolide resistance gene, in both animal (71.4%) and human isolates (41.8%), followed by *ermA*. Data on the prevalence of macrolide resistance genes in staphylococci from different animal species and from humans have been published[46,263,269,277,310,312,478]. Analysis of erythromycin resistance determinants in *S. aureus* of poultry and human origin from the USA has shown *ermA* to be predominant (100% in poultry and 88% in humans) followed by *erm*C (50%) in poultry and *erm*B (72%) in human isolates[310]. Nicola *et al* has also shown that the *ermA* gene was the predominant macrolide resistance gene of human *S. aureus* isolates originating from the USA between 1958 and 1969[312]. Both authors conclude that *ermC* has only recently become prevalent in the *S. aureus* population[140]. Also study by Westh *et al* has shown that while in human *S. aureus* in Denmark from the late 1950's *ermA* was solely responsible for erythromycin resistance by 1988 *ermC* had replaced *ermA* as

the dominant gene[478]. They also reported that the *ermA* and *ermC* genes were responsible for over 98% of erythromycin resistance in human blood culture isolates with the *ermB* gene infrequently found. These data contrast with those of Nawaz *et al* who have found the *erm*A gene to be the predominant erythromycin resistance gene carried in 72% of human clinical *S. aureus* collected in 1999 while *erm*C was carried in only 4% of these isolates[310]. Data published by Lüthje and Schwarz has shown that *ermB* was the predominant resistance gene among erythromycin resistant *S. aureus* isolated from domestic and companion animals collected in Germany between 2003 and 2005[277]. In contrast, in both isolate collections in this study the *ermB* gene was detected at a frequency of between 1.5-3.6%. In agreement with data from this study, investigations into the molecular mechanisms of macrolide resistance conducted by Weisblum and Schmitz *et al*, among *S. aureus* isolated from humans, and Jensen *et al* and Nawaz *et al*, among *S. aureus* isolated from domestic animals, have all shown a very low frequency of *ermB* gene detection[202,310,375,474].

It is interesting to note that while *ermA* has been shown to be the predominant resistance gene found in MRSA of human origin, followed by *erm*C, both *ermA* and *ermC* were found at an identical frequency among the human isolates of MRSA in this study[23,140,263]. This data contrasts with that of Spiliopoulou *et al* who found that *ermC* was most prevalent amongst human MRSA in Greece collected between 1999 and 2001, which they attribute to the spread of two major MRSA clones[409]. It is likely that the equal distribution of *ermA* and *ermC* genes among the human MRSA in this study is as reflection of the large number of different clonal backgrounds examined. The *ermA* gene was the most prevalent *erm* gene detected in companion animals, previously characterized as USA100 ST5, from the USA. It has been proposed that MRSA isolated

from companion animals originate from humans[270]. The MRSA USA100 clone is a common human MRSA and it is therefore likely that these isolates were of human origin. Kennedy *et al* have also shown *erm*A to be the most prevalent macrolide resistance gene found in human community acquired MRSA in the USA which they attribute to the recent emergence and expansion of the USA300 MRSA clone[223]. While in this study all *S. aureus* isolated from animals carried one of the *erm* genes, over one quarter of human *S. aureus* isolates were non-typable using primers for the *ermA*, *ermB* and *ermC* genes. Jensen *et al* have also reported the absence of *erm* genes among *S. aureus* from human clinical sources and staphylococci from cattle although at a much lower frequency[202]. As PCR for the *msrA*, *msrB*, *ereA*, and *ereB* genes was not performed in that study or this study it could not be ruled out that these genes may be present in the non-typable resistant isolates.

### 5.4.6 Erythromycin MIC of human and animal *S. aureus* isolates

The human *S. aureus* isolates exhibited a wide erythromycin MIC range with *erm* gene non-typable strains exhibiting the widest MIC range (32->256mg/l). Regardless of the class of *erm* gene present all animal strains exhibited an $MIC_{50}$ and $MIC_{90}$ of ≥256 mg/l. This high level macrolide resistance has been reported previously by Lüthje *et al* in CNS from cattle and *S. aureus* from human sources[276,277]. While the MIC range of the CNS from cattle was between 4-≥128 mg/l over 70% of these isolates had an MIC of ≥128 mg/l. Those isolates with high MIC values carried either *ermB* or *ermC* expressed both inducibly and constitutively[276,277]. The human isolates also carried mainly *erm* genes, expressed in most cases constitutively, which conferred erythromycin MIC values of ≥64 mg/l[277]. Spiliopoulou *et al* have also shown *erm* gene containing MSSA and MRSA isolated from humans exhibiting MICs to erythromycin in the range 8-

>1024 mg/l. In the same study they also noted that some *ermC* containing MSSA could be susceptible to erythromycin by disc diffusion using the NCCLS criteria and had MICs, of between 1-3 mg/l, well below the published breakpoint[409]. As not all strains in this study were examined for the presence of *ermC* it is possible that some strains exhibiting susceptibility to erythromycin, by disc diffusion, may carry copies of *ermC*.

### 5.4.7 Induction of clindamycin resistance in erythromycin resistant isolates

The results of this study have shown three distinct $MLS_B$ resistance phenotypes; inducible, constitutive and negative. Among the animal isolates all livestock and wild animals, regardless of *erm* gene, expressed constitutive resistance. While among the companion animals there was an equal distribution of constitutive and inducible resistance. This high level of constitutive resistance in staphylococci from livestock and companion animals has been reported previously by Lüthje and Schwarz and their results are in agreement with data from this study[276]. Resistance to 14, 15 and 16 membered lactone ring structures in macrolides is a characteristic of constitutive *erm* gene expression resulting in high macrolide MICs. The use of tylosin, a 16 member veterinary macrolide, therapeutically in animals may explain the high frequency of constitutive $MLS_B$ resistance seen among the animal strains. No 16 member macrolides are licensed for use in humans[201]. Therefore, it is likely that this accounts for the lack of constitutive macrolide resistance in the human isolates in this study. A study by Steward *et al* have also shown that the majority of human *S. aureus* carrying either *ermA* or *ermC* expressed inducible $MLS_B$ resistance while those carrying *ermB* resistance was constitutive[411]. These results are in concordance with data from this study where all human isolates carrying either *ermA* or *ermC* expressed an inducible $MLS_B$ phenotype and those carrying *ermB* expressed a constitutive resistance phenotype. Steward *et al*

also reported the presence of erythromycin resistance and clindamycin susceptibility, an $MLS_B$ negative phenotype, in 10% of the isolates tested. These isolates were shown to carry the *msrA* gene which encodes active drug efflux[411]. All *erm* gene non-typable isolates in this study also had the negative $MLS_B$ phenotype. PCR for the detection of the *msrA* gene was not performed. The lack of an *erm* gene together with a negative $MLS_B$ phenotype suggests the presence of an active efflux mechanism in these isolates.

# Chapter 6 Overall conclusions and future work

## 6.1 Conclusion

The hypothesis of this study is that antibiotic use in animals has influenced antibiotic resistance in human *S. aureus* strains. In order to investigate this hypothesis the similarity of clones, the frequency of antibiotic resistance, the resistance genes responsible for tetracycline and macrolide resistance and the possibility of resistance gene transfer between animal and human *S. aureus* was examined.

The first step in determining the similarities between animal and human *S. aureus* involved an evaluation of phenotypic and genotyping methods to ensure that all isolates could be typed and that these methods had a suitable level of discrimination.

Antibiogram and phage typing have been used to characterize *S. aureus* isolated from animals for 50 years. All isolates were typable by antibiogram and a discriminatory value of 88.2% was obtained. The poor discriminatory power of this method is attributed to the fact that two strains with no epidemiological link can have the same antibiogram while two isolates of the same clone can acquire and lose plasmid mediated antibiotic resistance and exhibit different phenotypes. Seventy six percent of animal isolates were typable by bacteriophage typing and a discriminatory value of 83.5% was obtained. Similar high levels of nontypability have been shown previously with bovine and human *S. aureus* isolates[459]. Due to the low discriminatory values of both methods and in the case of phage typing poor typeability these methods were unsuitable for characterizing *S. aureus* isolated from animals.

The PCR based typing method, RS-PCR, resolved 27 distinct banding patterns with a discriminatory value of 86.2%. Although all isolates could be typed by this method the low level of discrimination makes it unsuitable for characterizing animal *S. aureus* isolates.

PFGE, MLST and *spa* typing had a typeability value of 100% and were shown to be highly discriminatory with values of 93.1%, 91.4% and 95.4% respectively. A high level of concordance was demonstrated between these three typing methods. While *spa* typing does not have the resolving power of PFGE subtyping, it has several advantages in terms of speed, ease of use, ease of interpretation and standardization. Although *spa* typing proved to be superior in discriminatory power, PFGE is suitable tool for typing *S. aureus* isolated from animals in short term outbreak situations, providing results comparable to those obtained by MLST. Of the three DNA based techniques used MLST produced the lowest discriminatory value. This method sequences conserved core genes that encode essential proteins and is better suited for long term epidemiological and evolutionary studies. Tenover *et al*, has reported that no single typing method appears to be clearly superior in all cases. The current ability of *spa* typing to distinguish both molecularly and epidemiologically linked strains rapidly and easily makes it particularly well suited for typing *S. aureus* isolated from animals[423].

Based on MLST analysis animal *S. aureus* isolates belonged to 18 different clonal backgrounds. Four predominant clones, CC5, CC9, CC97 and CC814, accounted for 84% of the isolates. The CC5 clone was represented by isolates from chicken, cattle, partridge, pig and turkey from Grampian, Highland, Northern Ireland and Southwest Scotland. The CC814 clone was represented by avian isolates from Highland and

Northern Ireland. The CC9 clone was represented by isolates from cattle and pig from Grampian and Highland regions. The CC97 clone, with the exception of a single chicken isolate from Northern Ireland, was represented by isolates from cattle. Two bovine predominant clones, CC9 and CC97, have been isolated from cases of hospital infection but are not a significant source of human infection. However, CC5, is known to be the third most prevalent *S. aureus* clone in the UK[240], one of the five major hospital acquired *S. aureus* clones world wide and is the most prevalent clone in the USA and Japan.

Five further *S. aureus* clones in this study (CC15, CC20, CC22, CC30 and CC45) found in the animal isolate collection have also been isolated from humans. In addition to the CC5 above this finding is particularly noteworthy in light of the clinical importance of CC22 (EMRSA-15), CC30 (EMRSA-16) and CC45 (Berlin clone) as the three most common causes of nosocomial bacteraemia in the UK. The relatedness of the six clones found in both animal and human isolate collections was further supported by the analysis of 20 different virulence determinants.

Thirteen percent of animal isolates were susceptible to the 22 antibiotics tested and 73.4% resistant to two or fewer. The highest frequency of resistance was to penicillin (65.2%) followed by tetracycline (31.8), sulphamethioxazole (20.2%), ciprofloxacin (19.7%) and streptomycin (10.7%). Isolates of CC5 exhibited resistance to ciprofloxacin, clindamycin, erythromycin, penicillin, streptomycin, tobramycin and tylosin, CC15 to penicillin and tetracycline, CC22 to penicillin and rifampicin and CC30 to penicillin. Isolates belonging to CC45 were fully susceptible. No resistance to fusidic acid, gentamicin, kanamycin, linezolid, mupirocin, oxacillin,

quinupristin/dalfopristin, teicoplanin or vancomycin was detected. Therefore, *S. aureus* isolates of animal origin exhibit resistance to antibiotics commonly used in the treatment of human infection.

Currently there are no internationally recognised breakpoints for biocide susceptibility testing. This study has found that *S. aureus* isolated from animals in the UK have either not or to only a limited degree developed low level resistance to biocides commonly used for disinfection. The determination of low level resistance to benzalkonium chloride and chlorhexidine in this study was based on previously published MIC data by Suller and Russell and Cookson *et al*[78,417]. At present the clinical significance of low level biocide resistance in *S. aureus* is not clear.

Examining the genes underlying tetracycline resistance in both animal and human isolate collections showed an identical distribution of *tet* genes and in both cases *tet*K was predominant. This gene was carried by animal isolates belonging to CC5 and CC15. Furthermore RFLP of *tet*K amplicons, from animal and human isolates, produced indistinguishable restriction patterns. The observation of identical resistance genes in different clonal backgrounds and from different geographical locations suggests that horizontal transfer of the *tet*K gene, rather than convergent evolution, has occurred[315].

Examining the genes underlying macrolide resistance in both animal and human isolates collections *erm*C followed by *erm*A were predominant. This has previously been reported by Westh *et al* and Nicola *et al*[312,478]. Two bovine isolates belonging to CC5 carried the *erm*C gene and an isolate from a Phillips goose hawk, also CC5, carried the

*ermA* gene. This data shows that wild and livestock animals can carry *S. aureus* clones, commonly found in humans in the UK, that harbour the predominant macrolide resistance genes.

High frequency transfer of a 4.4kb *tetK* plasmid from a chicken *S. aureus*, CC5 clone, to a laboratory *S. aureus* strain (8325-4) was observed. Moreover, this transfer of plasmid mediated tetracycline was shown to be stably maintained.

In conclusion *spa* typing has been shown to be the most flexible and suitable method for typing *S. aureus* isolates from animals. *S. aureus* clones that cause serious infection in humans can be isolated from wild, livestock and companion animals. These clones exhibit resistance to antibiotics used to treat human infection, harbour the same tetracycline and macrolide resistance genes and are able to horizontally transfer the *tetK* gene. These data are therefore supportive of the hypothesis that animals represent an important reservoir of antibiotic resistant *S. aureus* with the ability for strain and antibiotic resistance gene transfer between animals and humans

## 6.2 Future work

Two observations from this study merit further investigation. Firstly, human *S. aureus* isolates have been shown to carry copies of the *ermC* gene but could be susceptible to erythromycin by disc diffusion testing and have MICs below the published breakpoint[409]. It would therefore be interesting to determine if this phenomenon could be found in *S. aureus* from animals, what mechanism is preventing the expression of this gene and if the MIC could be increased by exposure to macrolides.

Secondly, the *cfr* (chloramphenicol/florfenicol resistance) gene encodes a 23S rRNA methyltransferase that confers resistance to linezolid, an antibiotic used exclusively in humans. Two bovine isolates were resistant to chloramphenicol. It would be interesting to examine these animal isolates for the presence of the *cfr* gene and if present what affect this has on the linezolid MIC.

1. 1957. Bergey's Manual of Determinative Bacteriology. Williams and Wilkins Co, Baltimore.

2. 2000. Reducing the use of antimicrobial agents in animals and man. Journal of Medical Microbiology **49**:111-113.

3. DANMAP 2003, Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, foods and humans in Denmark. 2003.

4. **Aarestrup F.M. and H. Hasman**. 2004. Susceptibility of different bacterial species isolated from food animals to copper sulphate, zinc chloride and antimicrobial substances used for disinfection. Veterinary Microbiology 100:83-89

5. **Aarestrup, F. M., Y. Agerso, P. Ahrens, J. C. Ostergaard, J. M. Madsen, and L. B. Jensen**. 2000. Antimicrobial susceptibility and presence of resistance genes in staphylococci from poultry. Veterinary Microbiology **74**:353-364.

6. **Aarestrup, F. M., H. D. Larsen, N. H. Eriksen, C. S. Elsberg, and N. E. Jensen**. 1999. Frequency of alpha- and beta-haemolysin in *Staphylococcus aureus* of bovine and human origin. A comparison between pheno- and genotype and variation in phenotypic expression. Acta Pathologica, Microbiologica et Immunologica Scandinavica **107**:425-430.

7. **Aarestrup, F. M., H. C. Wegener, and V. T. Rosdahl**. 1995. Evaluation of phenotypic and genotypic methods for epidemiological typing of *Staphylococcus aureus* isolates from bovine mastitis in Denmark. Veterinary Microbiology **45**:139-150.

8. **Aarestrup, F. M.** 1999. Association between the consumption of antimicrobial agents in animal husbandry and the occurrence of resistant bacteria among food animals. International Journal of Antimicrobial Agents **12**:279-285.

9. **Aarestrup, F. M.** 2000. Characterization of glycopeptide-resistant *Enterococcus faecium* (GRE) from broilers and pigs in Denmark: Genetic evidence that persistence of GRE in pig herds is associated with coselection by resistance to macrolides. Journal of Clinical Microbiology **38**:2774-2777.

10. **Aarestrup, F. M. and L. B. Jensen**. 2002. Trends in antimicrobial susceptibility in relation to antimicrobial usage and presence of resistance genes in *Staphylococcus hyicus* isolated from exudative epidermitis in pigs. Veterinary Microbiology **89**:83-94.

11. **Aarestrup, F. M., A. M. Seyfarth, H. D. Emborg, K. Pedersen, R. S. Hendriksen, and F. Bager**. 2001. Effect of abolishment of the use of antimicrobial agents for growth promotion on occurrence of antimicrobial resistance in fecal enterococci from food animals in Denmark. Antimicrobial Agents and Chemotherapy **45**:2054-2059.

12. **Aires de Sousa, M., M. I. Crisostomo, I. Santos Sanches, J. S. Wu, J. Fuzhong, A. Tomasz, and H. de Lencastre**. 2003. Frequent recovery of a single clonal type of multidrug-resistant *Staphylococcus aureus* from patients in two hospitals in Taiwan and China. Journal of Clinical Microbiology **41**:159-163.

13. **Aires de Sousa, M. and H. Lencastre**. 2004. Bridges from hospitals to the laboratory: genetic portraits of methicillin-resistant *Staphylococcus aureus* clones. FEMS Immunology & Medical Microbiology **40**:101-111.

14. **Aires-de-Sousa, M., K. Boye, H. de Lencastre, A. Deplano, M. C. Enright, J. Etienne, A. Friedrich, D. Harmsen, A. Holmes, X. W. Huijsdens, A. M. Kearns, A. Mellmann, H. Meugnier, J. K. Rasheed, E. Spalburg, B. Strommenger, M. J. Struelens, F. C. Tenover, J. Thomas, U. Vogel, H. Westh, J. Xu, and W. Witte**. 2006. High interlaboratory reproducibility of DNA sequence-based typing of bacteria in a multicenter study. Journal of Clinical Microbiology **44**:619-621.

15. **Aires-de-Sousa, M., C. E. S. R. Parente, O. Vieira-da-Motta, I. C. F. Bonna, D. A. Silva, and H. de Lencastre**. 2007. Characterization of *Staphylococcus aureus* isolates from buffalo, bovine, ovine, and caprine milk samples collected in Rio de Janeiro State, Brazil. Applied and Environmental Microbiology **73**:3845-3849.

16. **Akineden, O., C. Annemuller, A. A. Hassan, C. Lammler, W. Wolter, and M. Zschock**. 2001. Toxin genes and other characteristics of *Staphylococcus aureus* isolates from milk of cows with mastitis. Clinical and Diagnostic Laboratory Immunology **8**:959-964.

17. **Amin, V. M. and N. F. Olson**. 1967. Factors affecting the resistance of *Staphylococcus aureus* to hydrogen peroxide treatments in milk. Applied Microbiology **15**:97-101.

18. **Amin, V. M. and N. F. Olson**. 1968. Influence of catalase activity on resistance of catalase-positive staphylococci to hydrogen peroxide. Applied Microbiology **16**:267-270.

19. **Amorim, M. L., N. A. Faria, D. C. Oliveira, C. Vasconcelos, J. C. Cabeda, A. C. Mendes, E. Calado, A. P. Castro, M. H. Ramos, J. M. Amorim, and H. de Lencastre**. 2007. Changes in the clonal nature and antibiotic resistance profiles in methicillin-resistant *Staphylococcus aureus* isolates associated with the spread of EMRSA-15 clone in a tertiary-care Portuguese Hospital. Journal of Clinical Microbiology **45**:2881-2888.

20. **Andrews, J. M.** 2001. BSAC standardized disc susceptibility testing method. Journal of Antimicrobial Chemotherapy **48**:43-57.

21. **Andrews, J. M. and for the BSAC Working Party on Susceptibility Testing.** 2005. BSAC standardized disc susceptibility testing method (version 4). Journal of Antimicrobial Chemotherapy **56**:60-76.

22. **Annemuller, C., C. Lammler, and M. Zschock**. 1999. Genotyping of *Staphylococcus aureus* isolated from bovine mastitis. Veterinary Microbiology **69**:217-224.

23. **Ardic.N., Ozyurt.M., Sareyyupoglu.B., and Haznedaroglu.T.** 2005. Investigation of erythromycin and tetracycline resistance in methicillin-resistant staphylococci. International Journal of Antimicrobial Agents **26**:213-218.

24. **Arias, C. A., M. Vallejo, J. Reyes, D. Panesso, J. Moreno, E. Castaneda, M. V. Villegas, B. E. Murray, and J. P. Quinn**. 2008. Clinical and microbiological aspects of linezolid resistance mediated by the *cfr* gene encoding a 23S rRNA methyltransferase. Journal of Clinical Microbiology **46**:892-896.

25. **Bae, W., K. N. Kaya, D. D. Hancock, D. R. Call, Y. H. Park, and T. E. Besser**. 2005. Prevalence and antimicrobial resistance of thermophilic *Campylobacter* spp. from cattle farms in Washington State. Applied and Environmental Microbiology **71**:169-174.

26. **Bager, F.** 2000. DANMAP: monitoring antimicrobial resistance in Denmark. International Journal of Antimicrobial Agents **14**:271-274.

27. **Bahl, M. I., S. J. Sorensen, L. H. Hansen, and T. R. Licht**. 2004. Effect of tetracycline on transfer and establishment of the tetracycline-inducible conjugative transposon Tn916 in the guts of gnotobiotic rats. Applied and Environmental Microbiology **70**:758-764.

28. **Bannerman, T. L., G. A. Hancock, F. C. Tenover, and J. M. Miller**. 1995. Pulsed-field gel electrophoresis as a replacement for bacteriophage typing of *Staphylococcus aureus*. Journal of Clinical Microbiology **33**:551-555.

29. **Barbosa, T. M. and S. B. Levy**. 2001. Antibiotic use and resistance: What lies beneath! APUA Newsletter **19**:1-3.

30. **Barza, M.** 2002. Potential mechanisms of increased disease in humans from antimicrobial resistance in food animals. Clinical Infectious Diseases **34**:S123-S125.

31. **Barza, M., S. Gorbach, and S. J. DeVincent**. 2002. Introduction. Clinical Infectious Diseases **34**:S71-S72.

32. **Beaber, J. W., B. Hochhut, and M. K. Waldor**. 2004. SOS response promotes horizontal dissemination of antibiotic resistance genes. Nature **427**:72-74.

33. **Becker, K., A. W. Friedrich, G. Lubritz, M. Weilert, G. Peters, and C. Von Eiff**. 2003. Prevalence of genes encoding pyrogenic toxin superantigens and exfoliative toxins among strains of *Staphylococcus aureus* isolated from blood and nasal specimens. Journal of Clinical Microbiology **41**:1434-1439.

34. **Becker, K., R. Roth, and G. Peters**. 1998. Rapid and specific detection of toxigenic *Staphylococcus aureus*: use of two multiplex PCR enzyme immunoassays for amplification and hybridization of staphylococcal enterotoxin genes, exfoliative toxin genes, and toxic shock syndrome toxin 1 gene. Journal of Clinical Microbiology **36**:2548-2553.

35. **Bertolatti, D., T. Hannelly, M. Bishop, E. French, and M. Marendoli**. 1996. *Staphylococcus aureus* in Western Australian poultry. International Journal of Environmental Health Research **6**:277-287.

36. **Beutin, L., S. Kaulfuss, S. Herold, E. Oswald, and H. Schmidt**. 2005. Genetic analysis of enteropathogenic and enterohemorrhagic *Escherichia coli* serogroup O103 strains by molecular typing of virulence and housekeeping genes and pulsed-field gel electrophoresis. Journal of Clinical Microbiology **43**:1552-1563.

37. **Bhattacharya, D., H. Carleton, C. J. Tsai, E. J. Baron, and F. Perdreau-Remington**. 2007. Differences in clinical and molecular characteristics of skin and soft tissue methicillin-resistant *Staphylococcus aureus* isolates between two hospitals in northern California. Journal of Clinical Microbiology **45**:1798-1803.

38. **Bismuth, R., R. Zilhao, H. Sakamoto, J. L. Guesdon, and P. Courvalin**. 1990. Gene heterogeneity for tetracycline resistance in *Staphylococcus* spp. Antimicrobial Agents and Chemotherapy **34**:1611-1614.

39. **Bjorland, J., T. Steinum, B. Kvitle, S. Waage, M. Sunde, and E. Heir**. 2005. Widespread distribution of disinfectant resistance genes among staphylococci of bovine and caprine origin in Norway. Journal of Clinical Microbiology **43**:4363-4368.

40. **Bjorland, J., M. Sunde, and S. Waage**. 2001. Plasmid-borne *smr* gene causes resistance to quaternary ammonium compounds in bovine *Staphylococcus aureus*. Journal of Clinical Microbiology **39**:3999-4004.

41. **Blahna, M. T., C. A. Zalewski, J. Reuer, G. Kahlmeter, B. Foxman, and C. F. Marrs**. 2006. The role of horizontal gene transfer in the spread of trimethoprim-sulfamethoxazole resistance among uropathogenic Escherichia coli in Europe and Canada. Journal of Antimicrobial Chemotherapy **57**:666-672.

42. **Blair, J. E. and R. E. O. Williams**. 1961. Phage typing of staphylococci. Bulletin of the World Health Organisation **24**:771-784.

43. **Blanc, D. S., M. J. Struelens, A. Deplano, R. De Ryck, P. M. Hauser, C. Petignat, and P. Francioli**. 2001. Epidemiological validation of pulsed-field gel electrophoresis patterns for methicillin-resistant *Staphylococcus aureus*. Journal of Clinical Microbiology **39**:3442-3445.

44. **Bocchini, C. E., K. G. Hulten, E. O. Mason, Jr., B. E. Gonzalez, W. A. Hammerman, and S. L. Kaplan**. 2006. Panton-valentine leukocidin genes are associated with enhanced inflammatory response and local disease in acute hematogenous *Staphylococcus aureus* osteomyelitis in children. Pediatrics **117**:433-440.

45. **Boerlin, P., A. Wissing, F. M. Aarestrup, J. Frey, and J. Nicolet**. 2001. Antimicrobial growth promoter ban and resistance to macrolides and vancomycin in enterococci from pigs. Journal of Clinical Microbiology **39**:4193-4195.

46. **Boerlin, P., A. P. Burnens, J. Frey, P. Kuhnert, and J. Nicolet**. 2001. Molecular epidemiology and genetic linkage of macrolide and aminoglycoside resistance in *Staphylococcus intermedius* of canine origin. Veterinary Microbiology **79**:155-169.

47. **Boerlin, P., P. Kuhnert, D. Hussy, and M. Schaellibaum**. 2003. Methods for identification of *Staphylococcus aureus* isolates in cases of bovine mastitis. Journal of Clinical Microbiology **41**:767-771.

48. **Bolan, N. S., M. A. Khan, J. Donaldson, D. C. Adriano, and C. Matthew**. 2003. Distribution and bioavailability of copper in farm effluent. The Science of the Total Environment **309**:225-236.

49. **Booth, M. C., L. M. Pence, P. Mahasreshti, M. C. Callegan, and M. S. Gilmore**. 2001. Clonal associations among *Staphylococcus aureus* isolates from various sites of infection. Infection and Immunity **69**:345-352.

50. **Bouallegue-Godet, O., Y. B. Salem, L. Fabre, M. Demartin, P. A. D. Grimont, R. Mzoughi, and F. X. Weill**. 2005. Nosocomial outbreak caused by *Salmonella enterica* serotype Livingstone producing CTX-M-27 extended-spectrum {beta}-lactamase in a neonatal unit in Sousse, Tunisia. Journal of Clinical Microbiology **43**:1037-1044.

51. **Bradley, A. J. and M. J. Green**. 2001. An investigation of the impact of intramammary antibiotic dry cow therapy on clinical coliform mastitis. Journal of Dairy Science **84**:1632-1639.

52. **Brakstad, O. G., K. Aasbakk, and J. A. Maeland**. 1992. Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the nuc gene. Journal of Clinical Microbiology **30**:1654-1660.

53. **Brown, N. L., S. R. Barrett, J. Camakaris, B. T. Lee, and D. A. Rouch**. 1995. Molecular genetics and transport analysis of the copper-resistance determinant (*pco*) from *Escherichia coli* plasmid pRJ1004. Molecular Microbiology **17**:1153-1166.

54. **Butaye, P., L. A. Devriese, and F. Haesebrouck**. 2003. Antimicrobial growth promoters used in animal feed: effects of less well known antibiotics on Gram-positive bacteria. Clinical Microbiology Reviews **16**:175-188.

55. **Butaye, P., G. B. Michael, S. Schwarz, T. J. Barrett, A. Brisabois, and D. G. White**. 2006. The clonal spread of multidrug-resistant non-typhi salmonella serotypes. Microbes and Infection **8**:1891-1897.

56. **Butler, E., K. A. Joiner, M. Malamy, J. C. Barlett, and F. P. Tally**. 1984. Transfer of tetracycline or clindamycin resistance among strains of *Bacteroides fragilis* in experimental abcesses. Journal of Infectious Diseases **150**:20-24.

57. **Butterworth, A., N. A. Reeves, D. Harbour, G. Werrett, and S. C. Kestin**. 2001. Molecular typing of strains of *Staphylococcus aureus* isolated from bone and joint lesions in lame broilers by random amplification of polymorphic DNA. Poultry Science **80**:1339-1343.

58. **Bywater, R., H. Deluyker, E. Deroover, A. de Jong, H. Marion, M. McConville, T. Rowan, T. Shryock, D. Shuster, V. Thomas, M. Valle, and J. Walters**. 2004. A European survey of antimicrobial susceptibility among zoonotic and commensal bacteria isolated from food-producing animals. Journal of Antimicrobial Chemotherapy **54**:744-754.

59. **Cabral, K. G., C. Lämmler, M. Zschöck, H. Langoni, M. E. P. de Sá, C. Victória, and A. V. Da Silva**. 2004. Pheno and genotyping of *Staphylococcus aureus*, isolated from bovine milk samples from São Paulo, Brazil. Canadian Journal of Microbiology **50**:901-909.

60. **Capita, R., C. Alonso-Calleja, M. C. Garcia-Fernandez, and B. Moreno**. 2002. Characterization of *Staphylococcus aureus* isolated from poultry meat in Spain. Poultry Science **81**:414-421.

61. **Caprioli, A., L. Busani, J. L. Martel, and R. Helmuth**. 2000. Monitoring of antibiotic resistance in bacteria of animal origin: epidemiological and microbiological methodologies. International Journal of Antimicrobial Agents **14**:295-301.

62. **Carrico, J. A., C. Silva-Costa, J. Melo-Cristino, F. R. Pinto, H. de Lencastre, J. S. Almeidia, and M. Ramirez**. 2006. Illustration of a common framework for relating multiple typing methods by application to macrolide-resistant *Streptococcus pyogenes*. Journal of Clinical Microbiology **44**:2524-2532.

63. **Carter, P. E., K. Begbie, and F. M. Thomson-carter**. 2003. Coagulase gene variants associated with distinct populations of *Staphylococcus aureus*. Epidemiology and Infection **130**:207-219.

64. **Casewell, M., C. Friis, E. Marco, P. McMullin, and I. Phillips**. 2003. The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. Journal of Antimicrobial Chemotherapy **52**:159-161.

65. **Chambers, H. F.** 2001. The changing epidemiology of *Staphylococcus aureus?* Emerging Infectious Diseases **7**:178-182.

66. **Chan, S. S. W., K. C. Ng, D. J. Lyon, W. L. Cheung, A. F. B. Cheng, and T. H. Rainer**. 2003. Acute bacterial gastroenteritis: a study of adult patients with positive stool cultures treated in the emergency department. Emergancy Medicine Journal **20**:335-338.

67. **Chang, S., D. M. Sievert, J. C. Hageman, M. L. Boulton, F. C. Tenover, F. P. Downes, S. Shah, J. T. Rudrik, G. R. Pupp, W. J. Brown, D. Cardo, S. K. Fridkin, and the Vancomycin-Resistant Staphylococcus aureus Investigative Team**. 2003. Infection with vancomycin-resistant *Staphylococcus aureus* containing the *vanA* resistance gene. New England Journal of Medicine **348**:1342-1347.

68. **Chapman, P. A., C. A. Siddons, A. T. Cerdan Malo, and M. A. Harkin**. 2000. A one year study of *Escherichia coli* O157 in raw beef and lamb products. Epidemiology and Infection **124**:207-213.

69. **Chaslus-Dancla, E., Y. Glupczynski, G. Gerbaud, M. Lagorce, J. P. Lafont, and P. Courvalin**. 1989. Detection of apramycin resistant enterobacteriaceae in hospital isolates. FEMS Microbiology Letters **61**:261-265.

70. **Chen, S., S. Zhao, D. G. White, C. M. Schroeder, R. Lu, H. Yang, P. F. McDermott, S. Ayers, and J. Meng**. 2004. Characterization of multiple-antimicrobial-resistant salmonella serovars isolated from retail meats. Applied and Environmental Microbiology **70**:1-7.

71. **Chesneau, O., A. Morvan, S. Aubert, and N. El Solh**. 2000. The value of rRNA gene restriction site polymorphism analysis for delineating taxa in the genus staphylococcus. International Journal of Systematic and Evolutionary Microbiology **50**:689-697.

72. **Chinivasagam, H. N., R. J. Thomas, K. Casey, E. McGahan, E. A. Gardener, M. Rafiee, and P. J. Blackall**. 2004. Microbiological status of piggery effluent from 13 piggeries in the south east Queensland region of Australia. journal of Applied Microbiology **97**:883-891.

73. **Chopra, I. and M. Roberts**. 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiology and Molecular Biology Reviews **65**:232-260.

74. **Col, N. F. and R. W. O'Connor**. 1996. Estimating world-wide current antibiotic usage: Report of task force 1. Reviews of Infectious Disease 9:S232–S243.

75. **Collignon, P.** 2004. Antibiotic growth promoters. Journal of Antimicrobial Chemotherapy **54**:272.

76. **Cook, R. R.** 1997. Antimicrobial resistance--use in veterinary and human medicine. Journal of Antimicrobial Chemotherapy **39**:435.

77. **Cooksey, D. A. and H. R. Azad**. 1992. Accumulation of copper and other metals by copper-resistant plant-pathogenic and saprophytic pseudomonads. Applied and Environmental Microbiology **58**:274-278.

78. Cookson, B. D., Bolton, M. C., and Platt, J. H. Chlorhexidine resistance in MRSA or just elevated MIC? an in vitro and in vivo assessment. Antimicrobial Agents and Chemotherapy 35, 1997-2002. 1991.

79. **Cookson, B. D., H. Farrelly, P. Stapleton, R. P. J. Garvey, and M. R. Price**. 1991. Transferable resistance to triclosan in MRSA. The Lancet **337**:1548-1549.

80. **Cookson, B. D., D. A. Robinson, A. B. Monk, S. Murchan, A. Deplano, R. de Ryck, M. J. Struelens, C. Scheel, V. Fussing, S. Salmenlinna, J. Vuopio-Varkila, C. Cuny, W. Witte, P. T. Tassios, N. J. Legakis, W. van Leeuwen, A. van Belkum, A. Vindel, J. Garaizar, S. Haeggman, B. Olsson-Liljequist, U. Ransjo, M. Muller-Premru, W. Hryniewicz, A. Rossney, B. O'Connell, B. D. Short, J. Thomas, S. O'Hanlon, and M. C. Enright**. 2007. Evaluation of molecular typing methods in characterizing a European collection of epidemic methicillin-resistant *Staphylococcus aureus* strains: the HARMONY Collection. Journal of Clinical Microbiology **45**:1830-1837.

81. **Cooper, J. E. and E. J. Feil**. 2004. Multilocus sequence typing – what is resolved? TRENDS in Microbiology **12**:373-377.

82. **Cooper, J. E. and E. J. Feil**. 2006. The phylogeny of *Staphylococcus aureus* - which genes make the best intra-species markers? Microbiology **152**:1297-1305.

83. **Couto, I., S. Pereira, M. Miragaia, I. S. Sanches, and H. de Lencastre**. 2001. Identification of clinical staphylococcal isolates from humans by internal transcribed spacer PCR. Journal of Clinical Microbiology **39**:3099-3103.

84. **Crampton, S. E., C. Gerke, N. F. Schnell, W. W. Nichols, and F. Götz**. 1999. The intracellular adhesion (*ica*) locus is present in *Staphylococcus aureus* and is required for biofilm formation. Infection and Immunity **67**:5427-5433.

85. **Craven, N., J. C. Anderson, and T. O. Jones**. 1986. Antimicrobial drug susceptibility of *Staphylococcus aureus* isolated from bovine mastitis. Veterinary Record **118**:290-291.

86. **Crisostomo, M. I., H. Westh, A. Tomasz, M. Chung, D. C. Oliveira, and H. de Lencastre**. 2001. The evolution of methicillin resistance in *Staphylococcus aureus*: Similarity of genetic backgrounds in historically early methicillin-susceptible and -resistant isolates and contemporary epidemic clones. Proceedings of the National Academy of Science **98**:9865-9870.

87. **Crowther, J., M. D. Wyer, M. Bradford, D. Kay, and C. A. Francis**. 2003. Modeling faecal indicator concentrations in large rural catchments using land use and topographic data. Journal of Applied Microbiology **94**:962-973.

88. **Cuteri, V., M. L. Marenzoni, R. Mazzolla, N. Tosti, L. Merletti, S. Arcioni, and C. Valente**. 2004. *Staphylococcus aureus*: study of genomic similarity of strains isolated in veterinary pathology using amplified fragment length polymorphism (AFLP). Comparative Immunology, Microbiology and Infectious Diseases **27**:247-253.

89. **Dahl, K. H., D. D. G. Mater, M. J. Flores, P. J. Johnsen, T. Midtvedt, G. Corthier, and A. Sundsfjord**. 2007. Transfer of plasmid and chromosomal glycopeptide resistance determinants occurs more readily in the digestive tract of mice than in vitro and exconjugants can persist stably in vivo in the absence of glycopeptide selection. Journal of Antimicrobial Chemotherapy **59**:478-486.

90. **Darányi, J. V.** 1926. Pathogenität und Einteilung der Staphylokokken. Zenteralblatt fur Bakteriologie **99**:74-79.

91. **Davison, A. L.** 1968. The characterisation of a Micrococcus (*Staphylococcus lactis*) sp I3 with an atypical teichoic acid in its cell wall. The Biochemical Journal **110**:557-558.

92. **Davison, J.** 1999. Genetic exchange between bacteria in the environment. Plasmid **42**:73-91.

93. **De Buyser, M. L., A. Morvan, S. Aubert, F. Dilasser, and N. El Solh**. 1992. Evaluation of a ribosomal RNA gene probe for the identification of species and subspecies within the genus staphylococcus. Journal of General Microbiology 138:389-399.

94. **de Gialluly, C., J. Loulergue, G. Bruant, L. Mereghetti, S. Massuard, N. van der Mee, A. Audurier, and R. Quentin**. 2003. Identification of new phages to type *Staphylococcus aureus* strains and comparison with a genotypic method. Journal of Hospital Infection **55**:61-67.

95. **de Neeling, A. J., M. J. M. van den Broek, E. C. Spalburg, M. G. Santen-Verheuvel, W. D. C. Dam-Deisz, H. C. Boshuizen, A. W. van de Giessen, E. van Duijkeren, and X. W. Huijsdens**. 2007. High prevalence of methicillin resistant *Staphylococcus aureus* in pigs. Veterinary Microbiology **122**:366-372.

96. **de Niederhausern, S., C. Sabia, P. Messi, E. Guerrieri, G. Manicardi, and M. Bondi**. 2007. *vanA*-Type vancomycin-resistant enterococci in equine and swine rectal swabs and in human clinical samples. Current Microbiology **55**:240-246.

97. **De Oliveira, A. P., J. L. Watts, S. A. Salmon, and F. M. Aarestrup**. 2000. Antimicrobial susceptibility of *Staphylococcus aureus* isolated from bovine mastitis in Europe and the United States. Journal of Dairy Science **83**:855-862.

98. **de Sousa, M. A., T. Conceicao, C. Simas, and H. de Lencastre**. 2005. Comparison of genetic backgrounds of methicillin-resistant and -susceptible *Staphylococcus aureus* isolates from Portuguese hospitals and the community. Journal of Clinical Microbiology **43**:5150-5157.

99. **Delsol, A. A., J. Sunderland, M. J. Woodward, L. Pumbwe, L. J. V. Piddock, and J. M. Roe**. 2004. Emergence of fluoroquinolone resistance in the native *Campylobacter coli* population of pigs exposed to enrofloxacin. Journal of Antimicrobial Chemotherapy **53**:872-874.

100. **Deresinski, S.** 2005. Methicillin-resistant *Staphylococcus aureus*: An evolutionary, epidemiologic, and therapeutic odyssey. Clinical Infectious Diseases **40**:562-573.

101. **Desmonts, M. H., F. Dufour-Gesbert, L. Avrain, and I. Kempf.** 2004. Antimicrobial resistance in *Campylobacter* strains isolated from French broilers before and after antimicrobial growth promoter bans. Journal of Antimicrobial Chemotherapy **54**:1025-1030.

102. **Devriese, L. A., J. I. Cruz Cloque, P. De Herdt, and F. Haesebrouck.** 1992. Identification and composition of the tonsillar and anal enterococcal and streptococcal flora of dogs and cats. Journal of Applied Bacteriology **73**:421-425.

103. **Devriese, L. A. and J. Hommez.** 1975. Epidemiology of methicillin-resistant *Staphylococcus aureus* in dairy herds. Research in Veterinary Science **19**:23-27.

104. **Diep, B. A., F. Perdreau-Remington, and G. F. Sensabaugh.** 2003. Clonal characterization of *Staphylococcus aureus* by multilocus restriction fragment typing, a rapid screening approach for molecular epidemiology. Journal of Clinical Microbiology **41**:4559-4564.

105. **Diep, B., H. Carleton, R. Chang, G. Sensabaugh, and F. Perdreau–Remington.** 2006. Roles of 34 virulence genes in the evolution of hospital and community associated strains of methicillin–resistant *Staphylococcus aureus*. The Journal of Infectious Diseases **193**:1495-1503.

106. **Dimitrova, D. J., L. D. Lashev, S. G. Yanev, and B. Pandova.** Pharmacokinetics of enrofloxacin in turkeys. Research in Veterinary Science **In Press, Corrected Proof**.

107. **Dinges, M. M., P. M. Orwin, and P. M. Schlievert.** 2000. Exotoxins of *Staphylococcus aureus*. Clinical Microbiology Reviews **13**:16-34.

108. **Doern, G. V.** 1982. Evaluation of a commercial latex agglutination test for identification of *Staphylococcus aureus*. Journal of Clinical Microbiology **15**:416-418.

109. **Dogan, B., Y. H. Schukken, C. Santisteban, and K. J. Boor**. 2005. Distribution of serotypes and antimicrobial resistance genes among *Streptococcus agalactiae* isolates from bovine and human hosts. Journal of Clinical Microbiology **43**:5899-5906.

110. **Doherty, N., K. Trzcinski, P. Pickerill, P. Zawadzki, and C. G. Dowson**. 2000. Genetic diversity of the *tet*(M) gene in tetracycline-resistant clonal lineages of *Streptococcus pneumoniae*. Antimicrobial Agents and Chemotherapy **44**:2979-2984.

111. **Dolzani, L., E. Tonin, C. Lagatolla, and C. Monti-Bragadin**. 1994. Typing of *Staphylococcus aureus* by amplification of the 16S-23S rRNA intergenic spacer sequences. FEMS Microbiology Letters **119**:167-173.

112. **dos, S. N., K. R. N. dos Santos, E. Gentilini, D. Sordelli, and M. d. C. de Freire Bastos**. 2002. Phenotypic and genetic characterisation of bacteriocin-producing strains of *Staphylococcus aureus* involved in bovine mastitis. Veterinary Microbiology **85**:133-144.

113. **DuPont, H. L. and J. H. Steele**. 1987. Use of antimicrobial agents in animal feeds: implications for human health. Reviews of Infectious Disease **9**:447-460.

114. **Edelstein, M., M. Pimkin, T. Dmitrachenko, V. Semenov, N. Kozlova, D. Gladin, A. Baraniak, and L. Stratchounski**. 2004. Multiple outbreaks of nosocomial salmonellosis in Russia and Belarus caused by a single clone of *Salmonella enterica* Serovar Typhimurium producing an extended-spectrum {beta}-lactamase. Antimicrobial Agents and Chemotherapy **48**:2808-2815.

115. **Ellington, M. J., R. Hope, M. Ganner, M. Ganner, C. East, G. Brick, and A. M. Kearns**. 2007. Is Panton-Valentine leucocidin associated with the pathogenesis of *Staphylococcus aureus* bacteraemia in the UK? Journal of Antimicrobial Chemotherapy **60**:402-405.

116. **Emborg, H. D., A. K. Ersboll, O. E. Heuer, and H. C. Wegener**. 2001. The effect of discontinuing the use of antimicrobial growth promoters on the productivity in the Danish broiler production. Preventive Veterinary Medicine **50**:53-70.

117. **Endo, Y., T. Yamada, K. Matsunaga, Y. Hayakawa, T. Kaidoh, and S. Takeuchi**. 2003. Phage conversion of exfoliative toxin A in *Staphylococcus aureus* isolated from cows with mastitis. Veterinary Microbiology **96**:81-90.

118. **Endtz, H. P., G. J. Ruijs, B. van Klingeren, W. H. Jansen, T. van der Reyden, and R. P. Mouton**. 1991. Quinolone resistance in campylobacter isolated from man and poultry following the introduction of fluoroquinolones in veterinary medicine. Journal of Antimicrobial Chemotherapy **27**:199-208.

119. **Engberg, J., F. M. Aarestrup, D. E. Taylor, P. Gerner-Smidt, and I. Nachamkins**. 2001. Quinolone and macrolide resistance in *Campylobacter jejuni* and *C. coli*: Resistance mechanisms and trends in human isolates. Emerging Infectious Diseases **7**:24-34.

120. **Engberg, J., J. Neimann, J. M. Neilsen, F. M. Aarestrup, and V. Fussing**. 2004. Quinolone-resistant Campylobacter infections: Risk factors and clinical consequences. Emerging Infectious Diseases **10**:1056-1063.

121. **Engberg, J., J. Neimann, E. M. Nielson, F. M. Aarestrup, and V. Fussing**. 2004. Quinolone resistant *Campylobacter* infections: Risk factors and clinical consequences. Emerging Infectious Diseases **10**:1056-1063.

122. **Enright, M. C., N. P. J. Day, C. E. Davies, S. J. Peacock, and B. G. Spratt**. 2000. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. Journal of Clinical Microbiology **38**:1008-1015.

123. **Enright, M. C.** 2003. The evolution of a resistant pathogen - the case of MRSA. Current Opinion in Pharmacology **3**:474-479.

124. **Enright, M. C., N. P. J. Day, C. E. Davies, S. J. Peacock, and B. G. Spratt.** 2000. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. Journal of Clinical Microbiology **38**:1008-1015.

125. **Enright, M. C., D. A. Robinson, G. Randle, E. J. Feil, H. Grundmann, and B. G. Spratt.** 2002. The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). Proceedings of the National Academy of Science **99**:7687-7692.

126. **Evangelisti, D. G., A. R. English, A. E. Girard, J. E. Lynch, and I. A. Solomons.** 1975. Influence of subtherapeutic levels of oxytetracycline on *Salmonella typhimurium* in swine, calves, and chickens. Antimicrobial Agents and Chemotherapy **8**:664-672.

127. **Evans, M. C. and H. C. Wegener.** 2003. Antimicrobial growth promoters and *Salmonella* spp., *Campylobacter* spp. in poultry and swine, Denmark. Emerging Infectious Diseases **9**:489-492.

128. **Falkiner, F. R.** 1998. The consequences of antibiotic use in horticulture. Journal of Antimicrobial Chemotherapy **41**:429-431.

129. **Faria, N. A., J. A. Carrico, D. C. Oliveira, M. Ramirez, and H. de Lencastre.** 2007. Analysis of typing methods for epidemiological surveillance of both methicillin-resistant and susceptible *Staphylococcus aureus*. Journal of Clinical Microbiology. **Corrected Proof.**

130. **Feibelkorn, K. R., S. A. Crawford, M. L. McElmeel, and J. H. Jorgensen.** 2003. Practicle disk diffusion method for detection of inducible clindamycin resistance in *Staphylococcus sureus* and coagulase-negative staphylococci. Journal of Clinical Microbiology. **41**:4740-4744.

131. **Feighner, S. D. and M. P. Dashkevicz.** 1987. Subtherapeutic levels of antibiotics in poultry feeds and their effects on weight gain, feed efficiency, and bacterial cholyltaurine hydrolase activity. Applied and Environmental Microbiology **53**:331-336.

132. **Feil, E. J., J. E. Cooper, H. Grundmann, D. A. Robinson, M. C. Enright, T. Berendt, S. J. Peacock, J. M. Smith, M. Murphy, B. G. Spratt, C. E. Moore, and N. P. J. Day.** 2003. How clonal is *Staphylococcus aureus*? Journal of Bacteriology **185**:3307-3316.

133. **Feil, E. J., B. C. Li, D. M. Aanensen, W. P. Hanage, and B. G. Spratt.** 2004. eBURST: Inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. Journal of Bacteriology **186**:1518-1530.

134. **Feinman, S. E.** 1998. Antibiotics in animal feed - drug resistance revisited. ASM News **64**:24-30.

135. **Fey, P. D., T. J. Safranek, M. E. Rupp, E. F. Dunne, E. Ribot, P. C. Iwen, P. A. Bradford, F. J. Angulo, and S. H. Hinrichs.** 2000. Ceftriaxone-resistant salmonella infection acquired by a child from cattle. New England Journal of Medicine **342**:1242-1249.

136. **Fitzgerald, J. R., W. J. Meaney, P. J. Hartigan, and C. J. Smyth.** 1997. Fine-structure molecular epidemiological analysis of *Staphylococcus aureus* recovered from cows. Epidemiology and Infection **119**:261-269.

137. **Fitzgerald, J. R., D. E. Sturdevant, S. M. Mackie, S. R. Gill, and J. M. Musser.** 2001. Evolutionary genomics of *Staphylococcus aureus*: Insights into the origin of methicillin-resistant strains and the toxic shock syndrome epidemic. Proceedings of the National Academy of Science **98**:8821-8826.

138. **Fitzgerald, K. A., A. Davies, and A. D. Russell.** 1991. Sensitivity and resistance of *Escherichia coli* and *Staphylococcus aureus* to chlorhexidine. Letters in Applied Microbiology **14**:33-36.

139. **Fluit, A. C.** 2005. Towards more virulent and antibiotic-resistant Salmonella? FEMS Immunology and Medical Microbiology **43**:1-11.

140. **Fluit, A. C., M. R. Visser, and F. J. Schmitz.** 2001. Molecular detection of antimicrobial resistance. Clinical Microbiology Reviews **14**:836-871.

141. **Forbes, B. A. and D. R. Schaberg.** 1983. Transfer of resistance plasmids from *Staphylococcus epidermidis* to *Staphylococcus aureus*: evidence for conjugative exchange of resistance. Journal of Bacteriology **153**:627-634.

142. **Fox, J. L.** 2001. Report says agricultural antibiotic use and impact on resistance are high. ASM News **67**:128-129.

143. **Fox, P. M., R. J. Lampen, K. S. Stumpf, G. L. Archer, and M. W. Climo.** 2006. Successful therapy of experimental endocarditis caused by vancomycin-resistant *Staphylococcus aureus* with a combination of vancomycin and {beta}-lactam antibiotics. Antimicrobial Agents and Chemotherapy **50**:2951-2956.

144. **Frei, A., D. Goldenberger, and M. Tauber.** 2001. Antimicrobial susceptibility of intestinal bacteria from Swiss poultry flocks before the ban of antimicrobial growth promoters. Systematic and Applied Microbiology **24**:116-121.

145. **Frost, A. J.** 1967. Phage typing *Staphylococcus aureus* from dairy cattle in Australia. Journal of Hygiene, London **65**:311-319.

146. **Fueyo, J. M., M. C. Mendoza, M. R. Rodicio, J. Muniz, M. A. Alvarez, and M. C. Martin.** 2005. Cytotoxin and pyrogenic toxin superantigen gene profiles of *Staphylococcus aureus* associated with subclinical mastitis in dairy cows and relationships with macrorestriction genomic profiles. Journal of Clinical Microbiology **43**:1278-1284.

147. **Garnier, F., K. Gambarotto, F. Denis, and M. C. Ploy**. 2004. Molecular study of vancomycin-resistant enterococci isolated from humans and from food in a cattle-rearing area of France. Journal of Antimicrobial Chemotherapy **54**:236-239.

148. **Garvis, S., J. M. Mei, J. Ruiz-Albert, and D. W. Holden**. 2002. *Staphylococcus aureus svr*A: a gene required for virulence and expression of the *agr* locus. Microbiology **148**:3235-3243.

149. **Gaudreau, C. and H. Gilbert**. 1998. Antimicrobial resistance of clinical strains of *Campylobacter jejuni* subsp. jejuni isolated from 1985 to 1997 in Quebec, Canada. Antimicrobial Agents and Chemotherapy **42**:2106-2108.

150. **Gebreyes, W. A. and C. Altier**. 2002. Molecular characterization of multidrug-resistant *Salmonella enterica* subsp. enterica Serovar Typhimurium isolates from swine. Journal of Clinical Microbiology **40**:2813-2822.

151. **Gennaro, M. L.** 1990. DNA replication and its regulation in the pT181 plasmid family, p. 183-195. *In*: R. P. Novick and R. A. Skurray (eds.), Molecular biology of the staphylococci. VCH Publishers, Inc, New York.

152. **Gentilini, E., G. Denamiel, P. Llorente, S. Godaly, M. Rebuelto, and O. DeGregorio**. 2000. Antimicrobial susceptibility of *Staphylococcus aureus* isolated from bovine mastitis in Argentina. Journal of Dairy Science **83**:1224-1227.

153. **Ghebremedhin, B., W. Konig, W. Witte, K. J. Hardy, P. M. Hawkey, and B. Konig**. 2007. Subtyping of ST22-MRSA-IV (Barnim epidemic MRSA strain) at a university clinic in Germany from 2002 to 2005. Journal of Medical Microbiology **56**:365-375.

154. **Gianneechini, R. E., C. Concha, and A. Franklin**. 2002. Antimicrobial susecptibility of udder pathogens isolated from dairy herds in Uruguay. ACTA Veterinaria Scandinavica **43**:31-41.

155. **Gilot, P., G. Lina, T. Cochard, and B. Poutrel**. 2002. Analysis of the genetic variability of genes encoding the RNA III-activating components Agr and TRAP in a population of *Staphylococcus aureus* strains isolated from cows with mastitis. Journal of Clinical Microbiology **40**:4060-4067.

156. **Gilot, P. and W. B. van Leeuwen**. 2004. Comparative analysis of *agr* locus diversification and overall genetic variability among bovine and human *Staphylococcus aureus* isolates. Journal of Clinical Microbiology **42**:1265-1269.

157. **Gomes, A. R., H. Westh, and H. de Lencastre**. 2006. Origins and evolution of methicillin-resistant *Staphylococcus aureus* clonal lineages. Antimicrobial Agents and Chemotherapy **50**:3237-3244.

158. **Goni, P., Y. Vergara, J. Ruiz, I. Albizu, J. Vila, and R. Gomez-Lus**. 2004. Antibiotic resistance and epidemiological typing of *Staphylococcus aureus* strains from ovine and rabbit mastitis. International Journal of Antimicrobial Agents **23**:268-272.

159. **Gonzalez, N., J. Romero, and R. T. Espejo**. 2003. Comprehensive detection of bacterial populations by PCR amplification of the 16S-23S rRNA spacer region. Journal of Microbiological Methods **55**:91-97.

160. **Goossens, H.** 1999. Vancomycin-resistant *Enterococcus faecium* derived from animals colonizing the human gut: the missing evidence? Clinical Microbiology and Infection **5**:64-66.

161. **Goossens, H., M. Ferech, R. Vander Stichele, and M. Elseviers**. Outpatient antibiotic use in Europe and association with resistance: a cross-national database study. The Lancet **365**:579-587.

162. **Gorbach, S. L.** 2001. Antimicrobial use in animal feed -- Time to Stop. New England Journal of Medicine **345**:1202-1203.

163. **Graham, P. L., III, S. X. Lin, and E. L. Larson**. 2006. A U.S. population-based survey of *Staphylococcus aureus* colonization. Annals of Internal Medicine **144**:318-325.

164. **Grave, K., E. Lingaas, M. Bangen, and M. Ronning**. 2003. Surveillance of the overall consumption of antibacterial drugs in humans, domestic animals and farmed fish in Norway in 1992 and 1996. Journal of Antimicrobial Chemotherapy **43**:243-252.

165. **Grundmann, H., S. Hori, M. C. Enright, C. Webster, A. Tami, E. J. Feil, and T. Pitt**. 2002. Determining the genetic structure of the natural population of *Staphylococcus aureus*: a comparison of multilocus sequence typing with pulsed-field gel electrophoresis, randomly amplified polymorphic DNA analysis, and phage typing. Journal of Clinical Microbiology **40**:4544-4546.

166. **Grundmann, H., M. ires-de-Sousa, J. Boyce, and E. Tiemersma**. Emergence and resurgence of meticillin-resistant *Staphylococcus aureus* as a public-health threat. The Lancet **368**:874-885.

167. **Grundmeier, M., M. Hussain, P. Becker, C. Heilmann, G. Peters, and B. Sinha**. 2004. Truncation of fibronectin-binding proteins in *Staphylococcus aureus* strain Newman leads to deficient adherence and host cell invasion due to loss of the cell wall anchor function. Infection and Immunity **72**:7155-7163.

168. **Guardabassi, L., S. Schwarz, and D. H. Lloyd**. 2004. Pet animals as reservoirs of antimicrobial-resistant bacteria. Journal of Antimicrobial Chemotherapy. **54**:321-332

169. **Guler, L., U. Ok, K. Gunduz, Y. Gulcu, and H. H. Hadimli**. 2005. Antimicrobial susceptibility and coagulase gene typing of *Staphylococcus aureus* isolated from bovine clinical mastitis cases in Turkey. Journal of Dairy Science **88**:3149-3154.

170. **Gupta, A., J. Fontana, C. Crowe, B. Bolstorff, A. Stout, S. Van Duyne, M. P. Hoekstra, J. M. Whichard, T. J. Barrett, and F. J. Angulo**. 2003. Emergence of multidrug-resistant *Salmonella enterica* serotype Newport infections resistant to expanded-spectrum cephalosporins in the United States. The Journal of Infectious Diseases **188**:1707-1716.

171. **Hal Jones, C., M. Tuckman, A. Howe, M. Orlowski, S. Mullen, K. Chan, and P. A. Bradford**. 2006. Diagnostic PCR analysis of the occurrence of methicillin and tetracycline resistance genes among *Staphylococcus aureus* isolates from phase 3 clinical trials of tigecycline for complicated skin and skin structure infections. Antimicrobial Agents and Chemotherapy **50**:505-510.

172. **Hallin, M., A. Deplano, O. Denis, R. De Mendonca, R. De Ryck, and M. J. Struelens**. 2007. Validation of Pulsed-Field Gel Electrophoresis and spa Typing for Long-Term, Nationwide Epidemiological Surveillance Studies of Staphylococcus aureus Infections. J.Clin.Microbiol **45**:127-133.

173. **Harmsen, D., H. Claus, W. Witte, J. Rothganger, H. Claus, D. Turnwald, and U. Vogel**. 2003. Typing of methicillin-resistant *Staphylococcus aureus* in a University hospital setting by using novel software for *spa* repeat determination and database management. Journal of Clinical Microbiology **41**:5442-5448.

174. **Hartmann, F. A., Trostle.S.S, and A. A. Klohnen**. 1997. Isolation of methicillin-resistant *Staphylococcus aureus* from a postoperative wound infection in a horse. Journal of the American Veterinary Medical Association **211**:590-592.

175. **Hartstein, A. I., V. H. Morthland, S. Eng, G. L. Archer, F. D. Schoenknecht, and A. L. Rashad**. 1989. Restriction enzyme analysis of plasmid DNA and bacteriophage typing of paired *Staphylococcus aureus* blood culture isolates. Journal of Clinical Microbiology **27**:1874-1879.

176. **Hasman, H. and F. M. Aarestrup**. 2002. *tcr*B, a gene conferring transferable copper resistance in *Enterococcus faecium*: Occurrence, transferability, and linkage to macrolide and glycopeptide resistance. Antimicrobial Agents and Chemotherapy **46**:1410-1416.

177. **Hauschild, T.** 2001. Phenotypic and genotypic identification of staphylococci isolated from wild small animals. Systematic and Applied Microbiology 24:411-416.

178. **Hebert, G. A., R. C. Cooksey, N. C. Clark, B. C. Hill, W. R. Jarvis, and C. Thornsberry**. 1988. Biotyping coagulase-negative staphylococci. Journal of Clinical Microbiology **26**:1950-1956.

179. **Heir, E., G. Sundheim, and A. L. Holck**. 1999. The *qacG* gene on plasmid pST94 confers resistance to quaternary ammonium compounds in staphylococci isolated from the food industry. Journal of Applied Microbiology **86**:388.

180. **Helmuth, R. and D. Protz**. 1997. How to modify conditions limiting resistance in bacteria in animals and other reservoirs. Clinical Infectious Diseases **24**:S136-S138.

181. **Hermans, K., L. A. Devriese, and F. Haesebrouck**. 2003. Rabbit staphylococcosis: difficult solutions for serious problems. Veterinary Microbiology **91**:57-64.

182. **Herrero, I. A., N. C. Issa, and R. Patel**. 2002. Nosocomial spread of linezolid-resistant, vancomycin-resistant *Enterococcus faecium*. New England Journal of Medicine **346**:867-869.

183. **Herron-Olson, L., J. R. Fitzgerald, J. M. Musser, and V. Kapur**. 2007. Molecular correlates of host specialization in *Staphylococcus aureus*. PLoS ONE **2**:e1120.

184. **Hershberger, E., S. F. Oprea, S. M. Donabedian, M. Perri, P. Bozigar, P. Bartlett, and M. J. Zervos**. 2005. Epidemiology of antimicrobial resistance in enterococci of animal origin. Journal of Antimicrobial Chemotherapy **55**:127-130.

185. **Heuer, O. E., A. M. Hammerum, P. Collignon, and H. C. Wegener**. 2006. Human health hazard from antimicrobial-resistant enterococci in animals and food. Clinical Infectious Diseases **43**:911-916.

186. **Hiramatsu, K., N. Aritaka, H. Hanaki, S. Kawasaki, Y. Hosoda, S. Hori, Y. Fukuchi, and I. Kobayashi**. 1997. Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin. Lancet **350**:1670-1673.

187. **Hiramatsu, K.** 2001. Vancomycin-resistant *Staphylococcus aureus*: a new model of antibiotic resistance. The Lancet Infectious Diseases **1**:147-155.

188. **Hiramatsu, K., L. Cui, M. Kuroda, and T. Ito**. 2001. The emergence and evolution of methicillin-resistant *Staphylococcus aureus*. Trends in Microbiology **9**:486-493.

189. **Ho, G., W. H. Campbell, and E. Carlson**. 1989. Ovine-associated *Staphylococcus aureus* protein with immunochemical similarity to toxic shock syndrome toxin 1. Journal of Clinical Microbiology **27**:210-212.

190. **Hoekstra, K. A. and R. J. L. Paulton**. 2002. Clinical prevalence and antimicrobial susceptibility of *Staphylococcus aureus* and *Staphylococcus intermedius* in dogs. Journal of Applied Microbiology **93**:406-413.

191. **Holtfreter, S., D. Grumann, M. Schmudde, H. T. T. Nguyen, P. Eichler, B. Strommenger, K. Kopron, J. Kolata, S. Giedrys-Kalemba, I. Steinmetz, W. Witte, and B. M. Broker**. 2007. Clonal distribution of superantigen genes in clinical *Staphylococcus aureus* isolates. Journal of Clinical Microbiology **45**:2669-2680.

192. **Howden, B. P., P. D. R. Johnson, P. B. Ward, T. P. Stinear, and J. K. Davies**. 2006. Isolates with low-level vancomycin resistance associated with persistent methicillin-resistant *Staphylococcus aureus* bacteremia. Antimicrobial Agents and Chemotherapy **50**:3039-3047.

193. **Huijsdens, X., B. van Dijke, E. Spalburg, M. Santen-Verheuvel, M. Heck, G. Pluister, A. Voss, W. Wannet, and A. de Neeling**. 2006. Community-acquired MRSA and pig-farming. Annals of Clinical Microbiology and Antimicrobials **5**:26.

194. **Hunter, J. E., M. Bennett, C. A. Hart, J. C. Shelly, and J. R. Walton**. 1994. Apramycin-resistant *Escherichia coli* isolated from pigs and a stockman. Epidemiology and Infection **112**:473-480.

195. **Hunter, P. R. and M. A. Gaston**. 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. Journal of Clinical Microbiology **26**:2465-2466.

196. **Huys, G., K. D'Haene, J. Van Eldere, A. von Holy, and J. Swings**. 2005. Molecular diversity and characterization of tetracycline-resistant *Staphylococcus aureus* isolates from a poultry processing plant. Applied and Environmental Microbiology **71**:574-579.

197. **Ip, M., R. W. H. Yung, T. K. Ng, W. K. Luk, C. Tse, P. Hung, M. Enright, and D. J. Lyon**. 2005. Contemporary methicillin-resistant *Staphylococcus aureus* clones in Hong Kong. Journal of Clinical Microbiology **43**:5069-5073.

198. **Irizarry, L., T. Merlin, J. Rupp, and J. Griffith**. 1996. Reduced susceptibility of methicillin resistant *Staphylococcus aureus* to cetylpyridinium chloride and chlorhexidine. Chemotherapy **42**:248-252.

199. **Ishihara, K., T. Kira, K. Ogikubo, A. Morioka, A. Kojima, M. Kijima-Tanaka, T. Takahashi, and Y. Tamura**. 2004. Antimicrobial susceptibilities of Campylobacter isolated from food-producing animals on farms (1999-2001): results from the Japanese Veterinary Antimicrobial Resistance Monitoring Program. International Journal of Antimicrobial Agents **24**:261-267.

200. **Jarraud, S., G. J. Lyon, A. M. S. Figueiredo, L. Gerard, F. Vandenesch, J. Etienne, T. W. Muir, and R. P. Novick**. 2000. Exfoliatin-producing strains define a fourth *agr* specificity group in *Staphylococcus aureus*. Journal of Bacteriology **182**:6517-6522.

201. **Jarraud, S., C. Mougel, J. Thioulouse, G. Lina, H. Meugnier, F. Forey, X. Nesme, J. Etienne, and F. Vandenesch**. 2002. Relationships between *Staphylococcus aureus* genetic background, virulence factors, *agr* groups (alleles), and human disease. Infection and Immunity **70**:631-641.

202. **Jensen, L. B., N. Frimodt-Moller, and F. M. Aarestrup**. 1999. Presence of *erm* gene classes in Gram-positive bacteria of animal and human origin in Denmark. FEMS Microbiology Letters **170**:151-158.

203. **Jensen, L. B.** 1998. Differences in the occurrence of two base pair variants of Tn1546 from vancomycin-resistant enterococci from humans, pigs, and poultry. Antimicrobial Agents and Chemotherapy **42**:2463-2464.

204. **Jensen, L. B., P. Ahrens, L. Dons, R. N. Jones, A. M. Hammerum, and F. M. Aarestrup**. 1998. Molecular analysis of Tn1546 in *Enterococcus faecium* isolated from animals and humans. Journal of Clinical Microbiology **36**:437-442.

205. **Jensen, M. A., J. A. Webster, and N. Straus**. 1993. Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. Applied and Environtal Microbiology **59**:945-952.

206. **Johnsen, P. J., J. I. Osterhus, H. Sletvold, M. Sorum, H. Kruse, K. Nielsen, G. S. Simonsen, and A. Sundsfjord**. 2005. Persistence of animal and human glycopeptide-resistant enterococci on two Norwegian poultry farms formerly exposed to avoparcin is associated with a widespread plasmid-mediated *vanA* element within a polyclonal *Enterococcus faecium* population. Applied and Environmental Microbiology **71**:159-168.

207. **Johnson, W. M., S. D. Tyler, E. P. Ewan, F. E. Ashton, D. R. Pollard, and K. R. Rozee**. 1991. Detection of genes for enterotoxins, exfoliative toxins, and toxic shock syndrome toxin 1 in *Staphylococcus aureus* by the polymerase chain reaction. Journal of Clinical Microbiology **29**:426-430.

208. **Johnston, A. M.** 1998. Use of antimicrobial drugs in veterinary practice. British Medical Journal **317**:665-667.

209. **Joo, Y. S., L. K. Fox, W. C. Davis, G. A. Bohach, and Y. H. Park**. 2001. *Staphylococcus aureus* associated with mammary glands of cows: genotyping to distinguish different strains among herds. Veterinary Microbiology **80**:131-138.

210. **Jordens, J. Z. and T. H. Pennington**. 1991. Characterization of *Neisseria meningitidis* isolated by ribosomal RNA gene restriction patterns and restriction endonuclease digestion of chromosomal DNA. Epidemiology and Infection **107**:253-262.

211. **Jorgensen, H. J., T. Mork, D. A. Caugant, A. Kearns, and L. M. Rorvik**. 2005. Genetic variation among *Staphylococcus aureus* strains from Norwegian bulk milk. Applied and Environmental Microbiology **71**:8352-8361.

212. **Kahl, B. C., A. Mellmann, S. Deiwick, G. Peters, and D. Harmsen**. 2005. Variation of the polymorphic region X of the protein A gene during persistent airway infection of cystic fibrosis patients reflects two independent mechanisms of genetic change in *Staphylococcus aureus*. Journal of Clinical Microbiology **43**:502-505.

213. **Kaida, S., T. Miyata, Y. Yoshizawa, H. Igarashi, and S. Iwanaga**. 1989. Nucleotide and deduced amino acid sequences of staphylocoagulase gene from *Staphylococcus aureus* strain 213. Nucleic Acids Research **17**:8871.

214. **Kaida, S., T. Miyata, Y. Yoshizawa, S. i. Kawabata, T. Morita, H. Igarashi, and S. Iwanaga**. 1987. Nucleotide sequence of the staphylocoagulase gene: Its unique COOH-terminal 8 tandem repeats. Journal of Biochemistry (Tokyo) **102**:1177-1186.

215. **Kanemitsu, K., H. Yamamoto, M. Takemura, and J. Shimada**. 2001. Relatedness between the coagulase gene 3'-end region and coagulase serotypes among *Staphylococcus aureus* strains. Microbiology and Immunology **45**:23-27.

216. **Kapur, V., W. M. Sischo, R. S. Greer, T. S. Whittam, and J. M. Musser**. 1995. Molecular population genetic analysis of *Staphylococcus aureus* recovered from cows. Journal of Clinical Microbiology **33**:376-380.

217. **Katayama, Y., D. A. Robinson, M. C. Enright, and H. F. Chambers**. 2005. Genetic background affects stability of *mec*A in *Staphylococcus aureus*. Journal of Clinical Microbiology **43**:2380-2383.

218. **Katsuda, K., E. Hata, H. Kobayashi, M. Kohmoto, K. Kawashima, H. Tsunemitsu, and M. Eguchi**. 2005. Molecular typing of *Staphylococcus aureus* isolated from bovine mastitic milk on the basis of toxin genes and coagulase gene polymorphisms. Veterinary Microbiology **105**:301-305.

219. **Kaulfers, P. M., H. Karch, and R. Laufs**. 1987. Plasmid-mediated formaldehyde resistance in *Serratia marcescens* and *Echerichia coli*: alterations in the cell wall. Zentralblatt für Bacteriologie Mikrobiologie und Hygiene **266**:239-248.

220. **Kawano, J., A. Shimizu, Y. Saitoh, M. Yagi, T. Saito, and R. Okamoto**. 1996. Isolation of methicillin-resistant coagulase-negative staphylococci from chickens. Journal of Clinical Microbiology **34**:2072-2077.

221. **Kelley, T. R., O. C. Pancorbo, W. C. Merka, and H. M. Barnharts**. 1998. Antibiotic resistance of bacterial litter isolates. Poultry Science **77**:243-247.

222. **Kelly, L., D. L. Smith, E. L. Snary, J. A. Johnson, A. D. Harris, M. Wooldridge, and J. Morris**. 2004. Animal growth promoters: to ban or not to ban?: A risk assessment approach. International Journal of Antimicrobial Agents **24**:205-212.

223. **Kennedy, A. D., M. Otto, K. R. Braughton, A. R. Whitney, L. Chen, B. Mathema, J. R. Mediavilla, K. A. Byrne, L. D. Parkins, F. C. Tenover, B. N. Kreiswirth, J. M. Musser, and F. R. DeLeo**. 2008. From the cover: Epidemic community-associated methicillin-resistant *Staphylococcus aureus*: Recent clonal expansion and diversification. Proceedings of the National Academy of Science **105**:1327-1332.

224. **Kenny, K., R. F. Reiser, F. D. Bastida-Corcuera, and N. L. Norcross**. 1993. Production of enterotoxins and toxic shock syndrome toxin by bovine mammary isolates of *Staphylococcus aureus*. Journal of Clinical Microbiology **31**:706-707.

225. **Khac, H. V., E. Holoda, E. Pilipcinec, M. Blanco, J. E. Blanco, A. Mora, G. Dahbi, C. Lopez, E. A. Gonzalez, and J. Blanco**. 2006. Serotypes, virulence genes, and PFGE profiles of *Escherichia coli* isolated from pigs with postweaning diarrhoea in Slovakia. BMC Veterinary Research **2**:10-18.

226. **Khachatourians, G. G.** 2004. Agricultural use of antibiotics and the evolution and transfer of antibiotic-resistant bacteria. Canadian Medical Association Journal **159**.

227. **Khan, S. A., R. R. Koepsel, R. W. Murray, and J. M. Zock**. 1990. In vitro replication of pT181 plasmid, p. 197-210. *In*: R. P. Novick (ed.), Molecular biology of the staphylococci. VCH Publishers Inc, New York.

228. **Khan, S. A. and R. P. Novick**. 1983. Complete nucleotide sequence of pT181, a tetracycline-resistance plasmid from *Staphylococcus aureus*. Plasmid **10**:251-259.

229. **Khanna, T., R. Friendship, C. Dewey, and J. S. Weese**. 2008. Methicillin resistant *Staphylococcus aureus* colonization in pigs and pig farmers. Veterinary Microbiology **128**:298-303.

230. **Kim, M. N., S. H. Hwang, Y. J. Pyo, H. M. Mun, and C. H. Pai**. 2002. Clonal spread of *Staphylococcus aureus* heterogeneously resistant to vancomycin in a University hospital in Korea. Journal of Clinical Microbiology **40**:1376-1380.

231. **King, M. D., B. J. Humphrey, Y. F. Wang, E. V. Kourbatova, S. M. Ray, and H. M. Blumberg**. 2006. Emergence of community-acquired methicillin-resistant *Staphylococcus aureus* USA 300 clone as the predominant cause of skin and soft-tissue infections. Annals of Internal Medicine **144**:309-317.

232. **Kirst, H. A., D. G. Thompson, and T. I. Nicas**. 1998. Historical yearly usage of vancomycin. Antimicrobial Agents and Chemotherapy **42**:1303-1304.

233. **Klare, I., H. Heier, H. Claus, G. Bohme, S. Marin, G. Seltmann, R. Hakenbeck, V. Antanassova, and W. Witte**. 1995. *Enterococcus faecium* strains with *vanA*-mediated high level glycopeptide resistance isolated from animal foodstuffs and fecal samples of humans in the community. Microbial Drug Resistance **1**:265-272.

234. **Kloos, W. E.** 1980. Natural populations of the genus *Staphylococcus*. Annual Reviews in Microbiology 34:559-592.

235. **Kloos, W. E., T. G. Tornabene, and K. H. Schleifer**. 1974. Identification and characterization of micrococci from human skin, including two new species:*Micrococcus lylae* and *Micrococcus kristinae* . International Journal of Systematic Bacteriology **24**:79-101.

236. **Kluytmans, J., A. van Belkum, and H. Verbrugh**. 1997. Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks. Clinical Microbiology Reviews **10**:505-520.

237. **Kluytmans, J., H. Berg, P. Steegh, F. Vandenesch, J. Etienne, and A. van Belkum**. 1998. Outbreak of *Staphylococcus schleiferi* wound infections: Strain characterization by randomly amplified polymorphic DNA analysis, PCR ribotyping, conventional ribotyping, and pulsed-field gel electrophoresis. Journal of Clinical Microbiology **36**:2214-2219.

238. **Kobayashi, N., H. Wu, K. Kojima, K. Taniquchi, S. Urasawa, N. Uehara, Y. Omizu, Y. Kishi, A. Yaqihashi, and I. Kurokawa**. 1994. Detection of *mecA*, *femA* and *femB* genes in clinical strains of staphylococci using polymerase chain reaction. Epidemiology and Infection **113**:259-266.

239. **Koreen, L., S. V. Ramaswamy, E. A. Graviss, S. Naidich, J. M. Musser, and B. N. Kreiswirth**. 2004. *spa* typing method for discriminating among *Staphylococcus aureus* isolates: Implications for use of a single marker to detect genetic micro- and macrovariation. Journal of Clinical Microbiology **42**:792-799.

240. **Koreen, L., S. V. Ramaswamy, S. Naidich, I. V. Koreen, G. R. Graff, E. A. Graviss, and B. N. Kreiswirth**. 2005. Comparative sequencing of the serine-aspartate repeat-encoding region of the clumping factor B gene (*clfB*) for resolution within clonal groups of *Staphylococcus aureus*. Journal of Clinical Microbiology **43**:3985-3994.

241. **Kuhn, G., P. Francioli, and D. S. Blanc**. 2006. Evidence for clonal evolution among highly polymorphic genes in methicillin-resistant *Staphylococcus aureus*. Journal of Bacteriology **188**:169-178.

242. **Kuhn, I., A. Iversen, M. Finn, C. Greko, L. G. Burman, A. R. Blanch, X. Vilanova, A. Manero, H. Taylor, J. Caplin, L. Dominguez, I. A. Herrero, M. A. Moreno, and R. Mollby**. 2005. Occurrence and relatedness of vancomycin-resistant enterococci in animals, humans, and the environment in different European regions. Applied and Environmental Microbiology **71**:5383-5390.

243. **Kumari, D. N., V. Keer, P. M. Hawkey, P. Parnell, N. Joseph, J. F. Richardson, and B. Cookson**. 1997. Comparison and application of ribosome spacer DNA amplicon polymorphisms and pulsed-field gel electrophoresis for differentiation of methicillin-resistant *Staphylococcus aureus* strains. Journal of Clinical Microbiology **35**:881-885.

244. **Kummerle, N., H. H. Feucht, and P. M. Kaulfers**. 1996. Plasmid-mediated formaldehyde resistance in *Escherichia coli*: characterization of resistance gene. Antimicrobial Agents and Chemotherapy **40**:2276-2279.

245. **Lange, C., M. Cardoso, D. Senczek, and S. Schwarz**. 1999. Molecular subtyping of *Staphylococcus aureus* isolates from cases of bovine mastitis in Brazil. Veterinary Microbiology **67**:127-141.

246. **Larsen, H. D., K. H. Sloth, C. Elsberg, C. Enevoldsen, L. H. Pedersen, N. H. R. Eriksen, F. M. Aarestrup, and N. E. Jensen**. 2000. The dynamics of *Staphylococcus aureus* intramammary infection in nine Danish dairy herds. Veterinary Microbiology **71**:89-101.

247. **Lathers, C. M.** 2001. Role of veterinary medicine in public health: Antibiotic use in food animals and humans and the effect on evolution of antibacterial resistance. Journal of Clinical Pharmacology **41**:595-599.

248. **Laval, A.** 2000. Veterinary use of antibiotics and resistance in man: what relation? Pathologie et Biologie (Paris) **48**:940-944.

249. **Layer, F., B. Ghebremedhin, W. Konig, and B. Konig**. 2006. Heterogeneity of methicillin-susceptible *Staphylococcus aureus* strains at a German University hospital implicates the circulating-strain pool as a potential source of emerging methicillin-resistant *S. aureus* clones. Journal of Clinical Microbiology **44**:2179-2185.

250. **Leclercq, R., J. Derlot, J. Duval, and P. Courvalin**. 1988. Plasmid-mediated resistance to vancomycin and teicoplanin in *Enterococcus faecium*. New England Journal of Medicine **319**:157-161.

251. **Lee, H. J., J. T. Suh, Y. S. Kim, W. Lenz, G. Bierbaum, and K. P. Schaal**. 2001. Typing and antimicrobial susceptibilities of methicillin resistant *Staphylococcus aureus* (MRSA) strains isolated in a hospital in Korea. Journal of Korean Medical Science **16**:381-385.

252. **Lee, J. C., S. Xu, A. Albus, and P. J. Livolsi**. 1994. Genetic analysis of type 5 capsular polysaccharide expression by *Staphylococcus aureus*. Journal of Bacteriology **176**:4883-4889.

253. **Lee, M. K. and A. J. Park**. 2001. Rapid species identification of coagulase negative staphylococci by rRNA spacer length polymorphism analysis. Journal of Infection **42**:189-194.

254. **Lee, P. K., B. N. Kreiswirth, J. R. Deringer, S. J. Projan, W. Eisner, J. N. Maslow, A. McGreer, D. E. Low, and R. P. Novick**. 1992. Nucleotide sequences and biological properties of toxic shock syndrome toxin 1 from ovine and bovine-associated *Staphylococcus aureus*. Journal of Infectious Diseases **165**:1056-1063.

255. **Leelaporn, A., I. T. Paulsen, J. M. Tennent, T. G. Littlejohn, and R. A. Skurray**. 1994. Multidrug resistance to antiseptics and disinfectants in coagulase negative staphylococci. Journal of Medical Microbiology **40**:214-220.

256. **Leonard, F. C., Y. Abbott, A. Rossney, P. J. Quinn, R. O'Mahony, and B. K. Markey**. 2006. Methicillin-resistant *Staphylococcus aureus* isolated from a veterinary surgeon and five dogs in one practice. Veterinary Record **158**:155.

257. **Leonard, R. B., J. Mayer, M. Sasser, M. L. Woods, B. R. Mooney, B. G. Brinton, P. L. Gayman, and K. C. Carroll**. 1995. Comparison of MIDI Sherlock system and pulsed-field gel electrophoresis in characterizing strains of methicillin-resistant *Staphylococcus aureus* from a recent hospital outbreak. Journal of Clinical Microbiology **33**:2723-2727.

258. **Lester, C. H., N. Frimodt-Moller, and A. M. Hammerum**. 2004. Conjugal transfer of aminoglycoside and macrolide resistance between *Enterococcus faecium* isolates in the intestine of streptomycin-treated mice. FEMS Microbiology Letters **235**:385-391.

259. **Levy, S. B. and B. Marshall**. 2004. Antibacterial resistance worldwide:Causes, challenges and responses. Nature medicine supplement **10**:S122-S129.

260. **Levy, S. B.** 1998. Antimicrobial resistance: bacteria on the defence. British Medical Journal **317**:612-613.

261. **Levy, S. B.** 1998. Multidrug resistance -- A sign of the times. New England Journal of Medicine **338**:1376-1378.

262. **Lina, G., Y. Piemont, F. Godail-Gamot, M. Bes, M.-O. Peter, V. Gauduchion, F. Vandenesch, and J. Etienne**. 1999. Involvment of Panton-Valentine leukocidin-producing *Staphylococcus aureus* in primary skin infections and pneumonia. Clinical Infectious Diseases **29**:1128-1132.

263. **Lina, G., A. Quaglia, M. E. Reverdy, R. Leclercq, F. Vandenesch, and J. Etienne**. 1999. Distribution of genes encoding resistance to macrolides, lincosamides, and streptogramins among staphylococci. Antimicrobial Agents and Chemotherapy **43**:1062-1066.

264. **Lindsay, J. A. and M. T. G. Holden**. 2004. *Staphylococcus aureus*: superbug, super genome? Trends in Microbiology **12**:378-385.

265. **Lindsay, J. A., C. E. Moore, N. P. Day, S. J. Peacock, A. A. Witney, R. A. Stabler, S. E. Husain, P. D. Butcher, and J. Hinds**. 2006. Microarrays reveal that each of the ten dominant lineages of *Staphylococcus aureus* has a unique combination of surface-associated and regulatory genes. Journal of Bacteriology **188**:669-676.

266. **Linton, A. H., K. Howe, P. M. Bennett, M. H. Richmond, and E. J. Whiteside**. 1977. The colonization of the human gut by antibiotic resistant Escherichia coli from chickens. Journal of Applied Bacteriology **43**:465-469.

267. **Littlejohn, T. G., I. T. Paulsen, M. T. Gillespie, J. M. Tennent, M. Midgley, I. G. Jones, A. S. Purewal, and R. A. Skurray**. 1992. Substrate specificity and energetics of antiseptic and disinfectant resistance in *Staphylococcus aureus*. FEMS Microbiology Letters **95**:259-265.

268. **Livermore, D. M.** 2001. Antibiotic resistance in staphylococci. International Journal of Antimicrobial Agents **16**:3-10.

269. **Lodder, G., C. Werckenthin, S. Schwarz, and K. Dyke**. 1997. Molecular analysis of naturally occurring *ermC*-encoding plasmids in staphylococci isolated from animals with and without previous contact with macrolide/lincosamide antibiotics. FEMS Immunology and Medical Microbiology **18**:7-15.

270. **Loeffler, A., A. K. Boag, J. Sung, J. A. Lindsay, L. Guardabassi, A. Dalsgaard, H. Smith, K. B. Stevens, and D. H. Lloyd**. 2005. Prevalence of methicillin-resistant *Staphylococcus aureus* among staff and pets in a small animal referral hospital in the UK. Journal of Antimicrobial Chemotherapy **56**:692-697.

271. **Lowy, F. D. and M. Miller**. 2002. New methods to investigate infectious disease transmission and pathogenesis -- *Staphylococcus aureus* disease in drug users. The Lancet Infectious Diseases **2**:605-612.

272. **Lu, H. Z., X. H. Weng, H. Li, Y. K. Yin, M. Y. Pang, and Y. W. Tang**. 2002. *Enterococcus faecium*-related outbreak with molecular evidence of transmission from pigs to humans. Journal of Clinical Microbiology **40**:913-917.

273. **Luber, P., J. Wagner, H. Hahn, and E. Bartelt**. 2003. Antimicrobial resistance in *Campylobacter jejuni* and *Campylobacter coli* strains isolated in 1991 and 2001-2002 from poultry and humans in Berlin, Germany. Antimicrobial Agents and Chemotherapy **47**:3825-3830.

274. **Luh, K. T., P. R. Hsueh, L. J. Teng, H. J. Pan, Y. C. Chen, J. J. Lu, J. J. Wu, and S. W. Ho**. 2000. Quinupristin-dalfopristin resistance among Gram-positive bacteria in Taiwan. Antimicrobial Agents and Chemotherapy **44**:3374-3380.

275. **Luong, T. T., S. Ouyang, K. Bush, and C. Y. Lee**. 2002. Type 1 capsule genes of *Staphylococcus aureus* are carried in a staphylococcal cassette chromosome genetic element. Journal of Bacteriology **184**:3623.

276. **Lüthje, P. and S. Schwarz**. 2006. Antimicrobial resistance of coagulase-negative staphylococci from bovine subclinical mastitis with particular reference to macrolide-lincosamide resistance phenotypes and genotypes. Journal of Antimicrobial Chemotherapy **57**:966-969.

277. **Lüthje, P. and S. Schwarz**. 2007. Molecular basis of resistance to macrolides and lincosamides among staphylococci and streptococci from various animal sources collected in the resistance monitoring program BfT-GermVet. International Journal of Antimicrobial Agents **In Press**.

278. **Luthje, P. and S. Schwarz.** 2006. Antimicrobial resistance of coagulase-negative staphylococci from bovine subclinical mastitis with particular reference to macrolide-lincosamide resistance phenotypes and genotypes. Journal of Antimicrobial Chemotherapy **57**:966-969.

279. **Malik, S., G. W. Coombs, F. G. O'Brien, H. Peng, and M. D. Barton.** 2006. Molecular typing of methicillin-resistant staphylococci isolated from cats and dogs. Journal of Antimicrobial Chemotherapy **58**:428-431.

280. **Manian, F. A.** 2003. Asymptomatic nasal carriage of mupirocin resistant MRSA in a pet dog associated with MRSA infection in household contacts. Clinical Infectious Diseases **36**:e26-e28.

281. **Mann, P. P.** 1959. Antibiotic sensitivity testing and bacteriophage typing of staphylococci found in the nostrils of dogs and cats. Journal of the American Veterinary Medical Association **134**:469-470.

282. **Marr, J. C., J. D. Lyon, J. R. Roberson, M. Lupher, W. C. Davis, and G. A. Bohach.** 1993. Characterization of novel type C staphylococcal enterotoxins: biological and evolutionary implications. Infection and Immunity **61**:4254-4262.

283. **Martineau, F., F. J. Picard, D. Ke, S. Paradis, P. H. Roy, M. Ouellette, and M. G. Bergeron.** 2001. Development of a PCR assay for identification of staphylococci at genus and species levels. Journal of Clinical Microbiology **39**:2541-2547.

284. **Mayrhofer, S., P. Paulsen, F. J. M. Smulders, and F. Hilbert.** 2004. Antimicrobial resistance profile of five major food-borne pathogens isolated from beef, pork and poultry. International Journal of Food Microbiology **97**:23-29.

285. **McBain, A. J., R. G. Ledder, L. E. Moore, C. E. Catrenich, and P. Gilbert.** 2004. Effects of quaternary-ammonium-based formulations on bacterial community dynamics and antimicrobial susceptibility. Applied and Environmental Microbiology **70**:3449-3456.

286. **McCullagh, J. J., P. T. McNamee, J. A. Smyth, and H. J. Ball.** 1998. The use of pulsed field gel electrophoresis to investigate the epidemiology of *Staphylococcus aureus* infection in commercial broiler flocks. Veterinary Microbiology **63**:275-281.

287. **McDonald, L. C., S. Rossiter, C. Mackinson, Y. Y. Wang, S. Johnson, M. Sullivan, R. Sokolow, E. DeBess, L. Gilbert, J. A. Benson, B. Hill, and F. J. Angulo.** 2001. Quinupristin-dalfopristin-resistant *Enterococcus faecium* on chicken and in human stool specimens. New England Journal of Medicine **345**:1155-1160.

288. **McDonnell, R. W., H. M. Sweeney, and S. Cohen.** 1983. Conjugational transfer of gentamicin resistance plasmids intra- and interspecifically in *Staphylococcus aureus* and *Staphylococcus epidermidis*. Antimicrobial Agents and Chemotherapy **23**:151-160.

289. **McEwen, S. A. and P. J. Fedorka-Cray.** 2002. Antimicrobial use and resistance in animals. Clinical Infectious Diseases **34 (Suppl 3)**:S93-S106.

290. **McKellar, Q. A.** 1998. Antimicrobial resistance: a veterinary perspective. British Medical Journal **317**:610-611.

291. **Mead, P. S., L. Slutsker, V. Dietz, L. F. McCraig, J. S. Bresee, C. Shapiro, P. Griffin, and R. V. Tauxe.** 1999. Food-related illness and death in the United States. Emerging Infectious Diseases **5**:607-625.

292. Melles, D. C., van Belkum, A., van Leeuwen, Willem, Verbrugh, H. A., and *et al.* Natural population dynamics and expansion of pathogenic clones of *Staphylococcus aureus*. The Journal of Clinical Investigation **114**(12), 1732-1740. 2004.

293. **Melles, D. C., W. B. van Leeuwen, S. V. Snijders, D. Horst-Kreft, J. K. Peeters, H. A. Verbrugh, and A. van Belkum**. 2007. Comparison of multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), and amplified fragment length polymorphism (AFLP) for genetic typing of *Staphylococcus aureus*. Journal of Microbiological Methods **69**:371-375.

294. **Menestrina, G., M. Dalla Serra, and G. Prevost**. 2001. Mode of action of [beta]-barrel pore-forming toxins of the staphylococcal [alpha]-hemolysin family. Toxicon **39**:1661-1672.

295. **Miranda, C. D., C. Kehrenberg, C. Ulep, S. Schwarz, and M. C. Roberts**. 2003. Diversity of tetracycline resistance genes in bacteria from Chilean salmon farms. Antimicrobial Agents and Chemotherapy **47**:883-888.

296. Mlot, C. Antimicrobials in Food Production: Resistance and Alternatives. ASM News **67**(4), 196-200. 2001.

297. **Molbak, K., D. L. Baggesen, F. M. Aarestrup, J. M. Ebbesen, J. Engberg, K. Frydendahl, P. Gerner-Smidt, A. M. Petersen, and H. C. Wegener**. 1999. An outbreak of multidrug-resistant, quinolone-resistant *Salmonella enterica* serotype Typhimurium DT104. New England Journal of Medicine **341**:1420-1425.

298. **Monday, S. R. and G. A. Bohach**. 1999. Use of multiplex PCR to detect classical and newly described pyrogenic toxin genes in staphylococcal Isolates. Journal of Clinical Microbiology **37**:3411-3414.

299. **Monecke, S., P. Kuhnert, H. Hotzel, P. Slickers, and R. Ehricht**. 2007 Microarray based study on virulence-associated genes and resistance determinants of Staphylococcus aureus isolates from cattle. Veterinary Microbiology **125**:128-140.

300. **Montesinos, I., E. Salido, T. Delgado, M. Cuervo, and A. Sierra**. 2002. Epidemiologic genotyping of methicillin-resistant *Staphylococcus aureus* by pulsed-field gel electrophoresis at a University hospital and comparison with antibiotyping and protein A and coagulase gene polymorphisms. Journal of Clinical Microbiology **40**:2119-2125.

301. **Moodley, A., M. Stegger, A. F. Bagcigil, K. E. Baptiste, A. Loeffler, D. H. Lloyd, N. J. Williams, N. Leonard, Y. Abbott, R. Skov, and L. Guardabassi**. 2006. *spa* typing of methicillin-resistant *Staphylococcus aureus* isolated from domestic animals and veterinary staff in the UK and Ireland. Journal of Antimicrobial Chemotherapy **58**:1118-1123.

302. **Moore, P. C. L. and J. A. Lindsay**. 2001. Genetic variation among hospital isolates of methicillin-sensitive *Staphylococcus aureus*: Evidence for horizontal transfer of virulence genes. Journal of Clinical Microbiology **39**:2760-2767.

303. **Moran, G. J., A. Krishnadasan, R. J. Gorwitz, G. E. Fosheim, L. K. McDougal, R. B. Carey, D. A. Talan, and the Emergency ID Net Study Group**. 2006. Methicillin-resistant *S. aureus* infections among patients in the emergency department. New England Journal Medicine **355**:666-674.

304. **Morandi, S., M. Brasca, R. Lodi, P. Cremonesi, and B. Castiglioni**. 2007. Detection of classical enterotoxins and identification of enterotoxin genes in *Staphylococcus aureus* from milk and dairy products. Veterinary Microbiology **124**:66-72.

305. **Mork, T., T. Tollersrud, B. Kvitle, H. J. Jorgensen, and S. Waage**. 2005. Comparison of *Staphylococcus aureus* genotypes recovered from cases of bovine, ovine, and caprine mastitis. Journal of Clinical Microbiology **43**:3979-3984.

306. **Moroni, P., G. Pisoni, M. Antonini, R. Villa, P. Boettcher, and S. Carli**. 2006. Short communication: Antimicrobial drug susceptibility of *Staphylococcus aureus* from subclinical bovine mastitis in Italy. Journal of Dairy Science **89**:2973-2976.

307. **Musser, J. M., P. M. Schlievert, A. W. Chow, P. Ewan, B. N. Kreiswirth, V. T. Rosdahl, A. S. Naidu, W. Witte, and R. K. Selander**. 1990. A single clone of *Staphylococcus aureus* causes the majority of cases of toxic shock syndrome. Proceedings of the National Academy of Science **87**:225-229.

308. **Na'was, T., A. Hawwari, E. Hendrix, J. Hebden, R. Edelman, M. Martin, W. Campbell, R. Naso, R. Schwalbe, and A. I. Fattom**. 1998. Phenotypic and genotypic characterization of nosocomial *Staphylococcus aureus* isolates from trauma patients. Journal of Clinical Microbiology **36**:414-420.

309. **Nagase, N., A. Sasaki, K. Yasashita, A. Shimizu, Y. Wakita, and S. Kitai**. 2002. Isolation and species distribution of Staphylococci from animal and human skin. Journal of Veterinary Medical Science **64**:245-250.

310. **Nawaz, M. S., S. A. Khan, A. A. Khan, F. M. Khambaty, and C. E. Cerniglia**. 2000. Comparative molecular analysis of erythromycin-resistance determinants in staphylococci isolates of poultry and human origin. Molecular CELL Probes **14**:311-319.

311. **Ng, L. K., I. Martin, M. Alfa, and M. Mulvey**. 2001. Multiplex PCR for the detection of tetracycline resistant genes. Molecular CELL Probes 15:209-215.

312. **Nicola, F. G., L. K. McDougal, J. W. Biddle, and F. C. Tenover**. 1998. Characterization of erythromycin-resistant isolates of *Staphylococcus aureus* recovered in the United States from 1958 through 1969. Antimicrobial Agents and Chemotherapy **42**:3024-3027.

313. **Nicoletti, G., V. Boghossian, F. Gurevitch, R. Borland, and P. Morgenroth**. 1993. The antimicrobial activity in vitro of chlorhexidine, a mixture of isothiazolinones ("Kathon" CG) and cetytrimethylammonium bromide (CTAB). Journal of Hospital Infection **23**:87-111.

314. **Nijsten, R., N. London, A. van den Bogaard, and E. Stobberingh**. 1995. In-vivo transfer of resistance plasmids in rat, human or pig-derived intestinal flora using a rat model. Journal of Antimicrobial Chemotherapy **36**:975-985.

315. **Nikolich, M. P., G. Hong, N. B. Shoemaker, and A. A. Salyers**. 1994. Evidence for natural horizontal transfer of *tet*Q between bacteria that normally colonize humans and bacteria that normally colonize livestock. Applied and Environmental Microbiology **60**:3255-3260.

316. **O'Brien, F. G., T. T. Lim, F. N. Chong, G. W. Coombs, M. C. Enright, D. A. Robinson, A. Monk, B. Said-Salim, B. N. Kreiswirth, and W. B. Grubb**. 2004. Diversity among community isolates of methicillin-resistant *Staphylococcus aureus* in Australia. Journal of Clinical Microbiology **42**:3185-3190.

317. **O'Gara, J. P.** 2007. *ica* and beyond: biofilm mechanisms and regulation in *Staphylococcus epidermidis* and *Staphylococcus aureus*. FEMS Microbiology Letters **270**:179-188.

318. **O'Mahony, R., Y. Abbott, F. C. Leonard, B. K. Markey, P. J. Quinn, P. J. Pollock, S. Fanning, and A. S. Rossney**. 2005. Methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from animals and veterinary personnel in Ireland. Veterinary Microbiology **109**:285-296.

319. **O'Riordan, C. and J. C. Lee**. 2004. *Staphylococcus aureus* Capsular Polysaccharides. Clinical Microbiology Reviews **17**:218-234.

320. **Ojeniyi, A. A.** 1989. Direct transmission of *Escherichia coli* from poultry to humans. Epidemiology and Infection **103**:513-522.

321. **Oliveira, D. C., A. Tomasz, and H. de Lencastre**. 2002. Secrets of success of a human pathogen: molecular evolution of pandemic clones of methicillin resistant *Staphylococcus aureus*. The Lancet Infectious Diseases **2**:180-189.

322. **Oliveira, D. C., I. Crisostomo, I. Santos-Sanches, P. Major, C. R. Alves, M. Aires de Sousa, M. K. Thege, and H. de Lencastre**. 2001. Comparison of DNA sequencing of the protein A gene polymorphic region with other molecular typing techniques for typing two epidemiologically diverse collections of methicillin-resistant *Staphylococcus aureus*. Journal of Clinical Microbiology **39**:574-580.

323. **Oliveira, M., R. Bexiga, S. F. Nunes, C. Carneiro, L. M. Cavaco, F. Bernardo, and C. L. Vilela**. 2006. Biofilm-forming ability profiling of *Staphylococcus aureus* and *Staphylococcus epidermidis* mastitis isolates. Veterinary Microbiology **118**:133-140.

324. **Olsen, S. J., E. E. DeBess, T. E. McGivern, N. Marano, T. Eby, S. Mauvais, V. K. Balan, G. Zirnstein, P. R. Cieslak, and F. J. Angulo**. 2001. A nosocomial outbreak of fluoroquinolone-resistant salmonella Infection. New England Journal of Medicine **344**:1572-1579.

325. **Oppegaard, H., T. M. Steinum, and Y. Wasteson**. 2001. Horizontal transfer of a multi-drug resistance plasmid between coliform bacteria of human and bovine origin in a farm environment. Applied and Environmental Microbiology **67**:3732-3734.

326. **Orden, J. A., J. A. Ruiz-Santa-Quiteria, D. Cid, R. Diez, S. Martinez, and R. de la Fuente**. 2001. Quinolone resistance in potentially pathogenic and non-pathogenic *Escherichia coli* strains isolated from healthy ruminants. Journal of Antimicrobial Chemotherapy **48**:421-424.

327. **Pai, M. P., K. A. Rodvold, P. C. Schreckenberger, R. D. Gonzalez, J. M. Petrolatti, and J. P. Quinn**. 2002. Risk factors associated with the development of infection with linezolid and vancomycin resistant *Enterococcus faecium*. Clinical Infectious Diseases **35**:1269-1272.

328. **Payot, S., S. Dridi, M. Laroche, M. Federighi, and C. Magras**. 2004. Prevalence and antimicrobial resistance of *Campylobacter coli* isolated from fattening pigs in France. Veterinary Microbiology **101**:91-99.

329. **Peacock, S. J., G. D. I. de Silva, A. Justice, A. Cowland, C. E. Moore, C. G. Winearls, and N. P. J. Day**. 2002. Comparison of multilocus sSequence typing and pulsed-field gel electrophoresis as tools for typing *Staphylococcus aureus* isolates in a microepidemiological setting. Journal of Clinical Microbiology **40**:3764-3770.

330. **Peacock, S. J., N. P. J. Day, M. G. Thomas, A. R. Berendt, and T. J. Foster**. 2000. Clinical isolates of *Staphylococcus aureus* exhibit diversity in *fnb* genes and adhesion to human fibronectin. Journal of Infection **41**:23-31.

331. **Peacock, S. J., A. Justice, D. Griffiths, G. D. I. de Silva, M. N. Kantzanou, D. Crook, K. Sleeman, and N. P. J. Day**. 2003. Determinants of acquisition and carriage of *Staphylococcus aureus* in infancy. Journal of Clinical Microbiology **41**:5718-5725.

332. **Peacock, S. J., C. E. Moore, A. Justice, M. Kantzanou, L. Story, K. Mackie, G. O'Neill, and N. P. J. Day**. 2002. Virulent combinations of adhesin and toxin genes in natural populations of *Staphylococcus aureus*. Infection and Immunity **70**:4987-4996.

333. **Perez-Roth, E., C. Lopez-Aguilar, J. coba-Florez, and S. Mendez-Alvarez.** 2006. High-level mupirocin resistance within methicillin-resistant *Staphylococcus aureus* pandemic lineages. Antimicrobial Agents and Chemotherapy **50**:3207-3211.

334. **Petersen, A., J. S. Andersen, T. Kaewmak, T. Somsiri, and A. Dalsgaard.** 2002. Impact of integrated fish farming on antimicrobial resistance in a pond environment. Applied and Environmental Microbiology **68**:6036-6042.

335. **Pezzella, C., A. Ricci, E. DiGiannatale, I. Luzzi, and A. Carattoli.** 2004. Tetracycline and streptomycin resistance genes, transposons, and plasmids in *Salmonella enterica* isolates from animals in Italy. Antimicrobial Agents and Chemotherapy **48**:903-908.

336. **Phillips, I., M. Casewell, T. Cox, B. De Groot, C. Friis, R. Jones, C. Nightingale, R. Preston, and J. Waddell.** 2004. Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. Journal of Antimicrobial Chemotherapy **53**:28-52.

337. **Piddock, L. J.** 1995. Quinolone resistance and *Campylobacter* spp. Journal of Antimicrobial Chemotherapy **36**:891-898.

338. **Piddock, L. J. V.** 2002. Fluoroquinolone resistance in salmonella serovars isolated from humans and food animals. FEMS Microbiology Reviews **26**:3-16.

339. **Pitt, T. L., M. A. Gaston, and P. N. Hoffman.** 1983. In vitro susceptibility of hospital isolates of various bacterial genera to chlorhexidine. Journal of Hospital Infection **4**:173-176.

340. **Poutrel, B. and L. Sutra.** 1993. Type 5 and 8 capsular polysaccharides are expressed by *Staphylococcus aureus* isolates from rabbits, poultry, pigs, and horses. Journal of Clinical Microbiology **31**:467-469.

341. **Pratt, A. and V. Korolik**. 2005. Tetracycline resistance of Australian *Campylobacter jejuni* and *Campylobacter coli* isolates. Journal of Antimicrobial Chemotherapy **55**:452-460.

342. **Prescott, J. F.** 1997. Antibiotics:miricle drugs or pig food? Canadian Veterinary Journal **38**:763-766.

343. **Pulverer, G.** 1986. Taxanomy of *Staphylococcus aureus*. Zentralblatt für Bacteriologie Mikrobiologie und Hygiene A262:425-437.

344. **Raimundo, O., M. Deighton, J. Capstick, and N. Gerraty**. 1999. Molecular typing of *Staphylococcus aureus* of bovine origin by polymorphisms of the coagulase gene. Veterinary Microbiology **66**:275-284.

345. **Rajala-Schultz, P. J., K. L. Smith, J. S. Hogan, and B. C. Love**. 2004. Antimicrobial susceptibility of mastitis pathogens from first lactation and older cows. Veterinary Microbiology **102**:33-42.

346. **Rankin, S., S. Roberts, K. O'Shea, D. Maloney, M. Lorenzo, and C. E. Benson**. 2005. Panton-Valentine leukocidin (PVL) toxin positive MRSA strains isolated from companion animals. Veterinary Microbiology **108**:145-148.

347. **Rankin, S. C., H. Aceto, J. Cassidy, J. Holt, S. Young, B. Love, D. Tewari, D. S. Munro, and C. E. Benson**. 2002. Molecular characterization of cephalosporin-resistant *Salmonella enterica* serotype Newport isolates from animals in Pennsylvania. Journal of Clinical Microbiology **40**:4679-4684.

348. **Rantala, M., E. Lahti, J. Kuhalampil, S. Pesonen, A. K. Jarvinen, Saijoumaa-Koulumies, and T. Honkanen-Buzalski**. 2004. Antibacteria resistance in *Staphylococcus* spp., *Escherichia coli* and *Enterococcus* spp. in dogs given antibiotics for chronic dermatological disorders, compared with non-treated control dogs. Acta Veterinaria Scandinavica **45**:37-45.

349. **Rimland, D. and B. Roberson**. 1986. Gastrointestinal carriage of methicillin-resistant *Staphylococcus aureus*. Journal of Clinical Microbiology **24**:137-138.

350. **Roberson, J. R., L. K. Fox, D. D. Hancock, J. M. Gay, and T. E. Besser.** 1994. Ecology of *Staphylococcus aureus* isolated from various sites on dairy farms. Journal of Dairy Science **77**:3354-3364.

351. **Roberts, M. C.** 1994. Epidemiology of tetracycline-resistance determinants. Trends in Microbiology **2**:353-357.

352. **Roberts, M. C.** 2004. Tetracycline therapy: Update. Clinical Infectious Diseases **36**:462-467.

353. **Roberts, M.** 1996. Tetracycline resistance determinants: mechanisms of action, regulation of expression, genetic mobility, and distribution. FEMS Microbiology Reviews **19**:1-24.

354. **Roberts, M. C., J. Sutcliffe, P. Courvalin, L. B. Jensen, J. Rood, and H. Seppala.** 1999. Nomenclature for macrolide and macrolide-lincosamide-streptogramin B resistance determinants. Antimicrobial Agents and Chemotherapy **43**:2823-2830.

355. **Robinson, D. A. and M. C. Enright.** 2004. Multilocus sequence typing and the evolution of methicillin-resistant *Staphylococcus aureus*. Clinical Microbiology and Infection **10**:92-97.

356. **Robinson, D. A. and M. C. Enright.** 2003. Evolutionary models of the emergence of methicillin-resistant *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy **47**:3926-3934.

357. **Robinson, D. A. and M. C. Enright.** 2004. Evolution of *Staphylococcus aureus* by large chromosomal replacements. Journal of Bacteriology **186**:1060-1064.

358. **Robinson, D. A., A. B. Monk, J. E. Cooper, E. J. Feil, and M. C. Enright.** 2005. Evolutionary genetics of the accessory gene regulator (*agr*) locus in *Staphylococcus aureus*. Journal of Bacteriology **187**:8312-8321.

359. **Robredo, B., K. V. Singh, F. Baquero, B. E. Murray, and C. Torres**. 1999. From *vanA Enterococcus hirae* to *vanA Enterococcus faecium*: a study of feed supplementation with avoparcin and tylosin in young chickens. Antimicrobial Agents and Chemotherapy **43**:1137-1143.

360. **Rodgers, J. D., J. J. McCullagh, P. T. McNamee, J. A. Smyth, and H. J. Ball**. 1999. Comparison of *Staphylococcus aureus* recovered from personnel in a poultry hatchery and in broiler parent farms with those isolated from skeletal disease in broilers. Veterinary Microbiology **69**:189-198.

361. **Rodgers, J. D., J. J. McCullagh, P. T. McNamee, J. A. Smyth, and H. J. Ball**. 2001. An investigation into the efficacy of hatchery disinfectants against strains of *Staphylococcus aureus* associated with the poultry industry. Veterinary Microbiology **82**:131-140.

362. **Sabat, A., N. Malachowa, J. Miedzobrodzki, and W. Hryniewicz**. 2006. Comparison of PCR-based methods for typing *Staphylococcus aureus* isolates. Journal of Clinical Microbiology **44**:3804-3807.

363. **Sabour, P. M., J. J. Gill, D. Lepp, J. C. Pacan, R. Ahmed, R. Dingwell, and K. Leslie**. 2004. Molecular typing and distribution of *Staphylococcus aureus* isolates in eastern Canadian dairy herds. Journal of Clinical Microbiology **42**:3449-3455.

364. **Saenz, Y., M. Zarazaga, M. Lantero, M. J. Gastanares, F. Baquero, and C. Torres**. 2000. Antibiotic resistance in *Campylobacter* strains isolated from animals, foods, and humans in Spain in 1997-1998. Antimicrobial Agents and Chemotherapy **44**:267-271.

365. **Sahm, D. F., J. Kissinger, M. S. Gilmore, P. R. Murray, R. Mulder, J. Solliday, and B. Clarke**. 1989. In vitro susceptibility studies of vancomycin-resistant *Enterococcus faecalis*. Antimicrobial Agents and Chemotherapy **33**:1588-1591.

366. **Sakurai, S., H. Suzuki, and K. Machida**. 1995. Rapid identification by polymerase chain reaction of staphylococcal exfoliative toxin serotypes A and B genes. Microbiology and Immunology **39**:379-386.

367. **Salauze, D., I. Otal, R. Gomez-Lus, and J. Davies**. 1990. Aminoglycoside acetyltransferase 3-IV (*aac*C4) and hygromycin B 4-I phosphotransferase (*hphB*) in bacteria isolated from human and animal sources. Antimicrobial Agents and Chemotherapy **34**:1915-1920.

368. **Salyers, A. A., A. Gupta, and Y. Wang**. 2004. Human intestinal bacteria as reservoirs for antibiotic resistance genes. Trends in Microbiology **12**:412-416.

369. **Sasatsu, M., K. Shimizu, N. Noquchi, and M. Kono**. 1993. Triclosan resistant *Staphylococcus aureus*. Lancet **341**:756.

370. **Schlegelova, J., M. Dendis, J. Benedik, V. Babak, and D. Rysanek**. 2003. *Staphylococcus aureus* isolates from dairy cows and humans on a farm differ in coagulase genotype. Veterinary Microbiology **92**:327-334.

371. **Schleifer, K. H. and O. Kandler**. 1972. The peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriology Reviews **36**:407-477.

372. **Schlievert, P. M., L. M. Jablonski, M. Roggiani, I. Sadler, S. Callantine, D. T. Mitchell, D. H. Ohlendorf, and G. A. Bohach**. 2000. Pyrogenic toxin superantigen site specificity in toxic shock syndrome and food poisoning in animals. Infection and Immunity **68**:3630-3634.

373. **Schmidt, A. S., M. S. Bruun, I. Dalsgaard, K. Pedersen, and J. L. Larsen**. 2000. Occurrence of antimicrobial resistance in fish-pathogenic and environmental bacteria associated with four Danish rainbow trout farms. Applied and Environmental Microbiology **66**:4908-4915.

374. **Schmitz, F. J., A. Krey, R. Sadurski, J. Verhoef, D. Milatovic, A. C. Fluit, and for the European SENTRY participants**. 2001. Resistance to tetracycline and distribution of tetracycline resistance genes in European *Staphylococcus aureus* isolates. Journal of Antimicrobial Chemotherapy **47**:239-240.

375. **Schmitz, F. J., R. Sadurski, A. Kray, M. Boos, R. Geisel, K. Kohrer, J. Verhoef, and A. C. Fluit**. 2000. Prevalence of macrolide-resistance genes in *Staphylococcus aureus* and *Enterococcus faecium* isolates from 24 European university hospitals. Journal of Antimicrobial Chemotherapy **45**:891-894.

376. **Schnabel, E. L. and A. L. Jones**. 1999. Distribution of tetracycline resistance genes and transposons among phylloplane bacteria in Michigan apple orchards. Applied and Environmental Microbiology **65**:4898-4907.

377. **Schouten, M. A., A. Voss, and J. A. Hoogkamp-Korstanje**. 1997. VRE and meat. Lancet **349**:1258.

378. **Schreckenberger, P. C., E. Ilendo, and K. L. Ristow**. 2004. Incidence of constitutive and inducible clindamycin resistance in *Staphylococcus aureus* and coagulase-negative staphylococci in a community and a tertiary care hospital. Journal of Clinical Microbiology **42**:2777-2779.

379. **Schroder, A., B. Schroder, B. Roppenser, S. Linder, B. Sinha, R. Fassler, and M. Aepfelbacher**. 2006. *Staphylococcus aureus* fibronectin binding protein-A induces motile attachment sites and complex actin remodeling in living endothelial cells. Molecular Biology of the Cell **17**:5198-5210.

380. **Schroeder, C. M., D. G. White, B. Ge, Y. Zhang, P. F. McDermott, S. Ayers, S. Zhao, and J. Meng**. 2003. Isolation of antimicrobial-resistant *Escherichia coli* from retail meats purchased in Greater Washington, DC, USA. International Journal of Food Microbiology **85**:197-202.

381. **Schuytema, E. C., H. L. Glenn, and C. C. Doughty**. 1969. Relative rates of lysis of staphylococcal cell walls by lytic enzymes from various bacteriophage types. Journal of Bacteriology **98**:923.

382. **Schwarz, S., M. Cardoso, and H. Blobel**. 1989. Plasmid-mediated Chloramphenicol Resistance in *Staphylococcus hyicus*. Journal of General Microbiology **135**:3329-3336.

383. **Schwarz, S. and W. C. Noble**. 1994. Tetracycline resistance genes in staphylococci from the skin of pigs. Journal of Applied Bacteriology **76**:320-326.

384. **Schwarz, S., M. C. Roberts, C. Werckenthin, Y. Pang, and C. Lange**. 1998. Tetracycline resistance in *Staphylococcus spp.* from domestic animals. Veterinary Microbiology **63**:217-227.

385. **Schwarz, S., C. Kehrenberg, and T. R. Walsh**. 2001. Use of antimicrobial agents in veterinary medicine and food animal production. International Journal of Antimicrobial Agents **17**:431-437.

386. **Schwarzkopf, A. and H. Karch**. 1994. Genetic variation in *Staphylococcus aureus* coagulase genes: potential and limits for use as epidemiological marker. Journal of Clinical Microbiology **32**:2407-2412.

387. **Seguin, J. C., R. D. Walker, J. P. Caron, W. E. Kloos, C. G. George, R. J. Hollis, R. N. Jones, and M. A. Pfaller**. 1999. Methicillin-resistant *Staphylococcus aureus* outbreak in a veterinary teaching hospital: Potential human-to-animal transmission. Journal of Clinical Microbiology **37**:1459-1463.

388. **Sekiguchi, J.-I., T. Fujino, K. Saruta, F. Kawano, J.-I. Takami, H. Miyazaki, T. Kuratsuji, H. Yoshikura, and T. Kirikae**. 2003. Spread of erythromycin-, tetracycline-, and aminoglycoside resistant genes in methicillin-resistant *Staphylococcus aureus* clinical isolates in a Kumamoto hospital. Japanease Journal of Infectious Diseases **56**:133-137.

389. **Seo, K. S., J. Y. Lim, H. S. Yoo, W. K. Bae, and Y. H. Park**. 2005. Comparison of vancomycin-resistant enterococci isolates from human, poultry and pigs in Korea. Veterinary Microbiology **106**:225-233.

390. **Shaw, C., J. M. Stitt, and S. T. Cowan**. 1951. Staphylococci and their classification. Journal of General Microbiology 5:1010-1023.

391. **Shea, K. M.** 2003. Antibiotic resistance: What is the impact of agricultural uses of antibiotics on children's health? Pediatrics **112**:253-258.

392. **Shenkman, B., E. Rubinstein, A. L. Cheung, G. E. Brill, R. Dardik, I. Tamarin, N. Savion, and D. Varon**. 2001. Adherence properties of *Staphylococcus aureus* under static and flow conditions: Roles of *agr* and *sar* loci, platelets, and plasma ligands. Infection and Immunity **69**:4473-4478.

393. **Shimizu, A., J. Ozaki, J. Kawano, Y. Saitoh, and S. Kimura**. 1992. Distribution of *Staphylococcus* species on animal skin. Journal of Veterinary Medical Science **54**:355-357.

394. **Shoemaker, N. B., H. Vlamakis, K. Hayes, and A. A. Salyers**. 2001. Evidence for extensive resistance gene transfer among *Bacteroides spp.* and among bacteroides and other genera in the human colon. Applied and Environmental Microbiology **67**:561-568.

395. **Shooter, R. A., E. M. Cooke, S. O'Farrell, K. A. Bettelheim, M. E. Chandler, and F. M. Bushrod**. 1974. The isolation of *Escherichia coli* from a poultry packing station and an abattoir. Journal of Hygiene, Cambridge **73**:245-247.

396. **Shopsin, B., M. Gomez, S. O. Montgomery, D. H. Smith, M. G. Waddington, D. E. Dodge, D. A. Bost, M. Riehman, S. Naidich, and B. N. Kreiswirth**. 1999. Evaluation of protein A gene polymorphic region DNA sequencing for typing of *Staphylococcus aureus* strains. Journal of Clinical Microbiology **37**:3556-3563.

397. **Sidhu, M. S., E. Heir, T. Leegaard, K. Wiger, and A. Holck**. 2002. Frequency of disinfectant resistance genes and genetic linkage with {beta}-lactamase transposon Tn552 among clinical staphylococci. Antimicrobial Agents and Chemotherapy **46**:2797-2803.

398. **Silva, E. R., J. U. D. Boechat, and N. Silva**. 2006. Coagulase gene polymorphism of *Staphylococcus aureus* isolated from goat mastitis in Brazilian dairy herds. Letters in Applied Microbiology **42**:30-34.

399. **Slanetz, L. W. and C. H. Bartley**. 1962. Bacteriophage and serological typing of staphylococci from bovine mastitis. Journal of Infectious Diseases **110**:238-245.

400. **Smith, E. M., L. E. Green, G. F. Medley, H. E. Bird, and C. G. Dowson**. 2005. Multilocus sequence typing of *Staphylococcus aureus* isolated from high-somatic-cell-count cows and the environment of an organic dairy farm in the United Kingdom. Journal of Clinical Microbiology **43**:4731-4736.

401. **Smith, E. M., L. E. Green, G. F. Medley, H. E. Bird, L. K. Fox, Y. H. Schukken, J. V. Kruze, A. J. Bradley, R. N. Zadoks, and C. G. Dowson**. 2005. Multilocus sequence typing of intercontinental bovine *Staphylococcus aureus* isolates. Journal of Clinical Microbiology **43**:4737-4743.

402. **Smith, H. W.** 1969. Transfer of antibiotic resistance from animal and human strains of *Escherichia coli* to resident *E. coli* in the alimentary tract of man. Lancet **i**:1174-1176.

403. **Smith, K. E., J. M. Besser, C. W. Hedberg, F. T. Leano, J. B. Bender, J. H. Wicklund, B. P. Johnson, K. A. Moore, M. T. Osterholm, and The Investigation Team**. 1999. Quinolone-resistant *Campylobacter jejuni* infections in Minnesota, 1992-1998. New England Journal of Medicine **340**:1525-1532.

404. **Sola, C., P. Cortes, H. A. Saka, Cordoba MRSA Collaborative Study Group, A. Vindel, and J. L. Bocco**. 2006. Evolution and molecular characterization of methicillin-resistant *Staphylococcus aureus* epidemic and sporadic clones in Cordoba, Argentina. Journal of Clinical Microbiology **44**:192-200.

405. **Sommerhauser, J., B. Kloppert, W. Wolter, M. Zschock, A. Sobiraj, and K. Failing**. 2003. The epidemiology of *Staphylococcus aureus* infections from subclinical mastitis in diary cows during a control programme. Veterinary Microbiology **96**:91-102.

406. **Song, J. Y., I. S. Hwang, J. S. Eom, H. J. Cheong, W. K. Bae, Y. H. Park, and W. J. Kim**. 2005. Prevalence and molecular epidemiology of vancomycin-resistant enterococci (VRE) strains isolated from animals and humans in Korea. Korean Journal of Internal Medicine **20**:55-62.

407. **Sordelli, D. O., F. R. Buzzola, M. I. Gomez, L. Steele-Moore, D. Berg, E. Gentilini, M. Catalano, A. J. Reitz, T. Tollersrud, G. Denamiel, P. Jeric, and J. C. Lee**. 2000. Capsule expression by bovine isolates of *Staphylococcus aureus* from Argentina: Genetic and epidemiologic analyses. Journal of Clinical Microbiology **38**:846-850.

408. **Spika, J. S., S. H. Waterman, G. W. Hoo, M. E. St Louis, R. E. Pacer, S. M. James, M. L. Bissett, L. W. Mayer, J. Y. Chiu, and B. Hall**. 1987. Chloramphenicol-resistant *Salmonella newport* traced through hamburger to dairy farms. A major persisting source of human salmonellosis in California. New England Journal of Medicine **316**:565-570.

409. **Spiliopoulou, I., E. Petinaki, P. Papandreou, and G. Dimitracopoulos**. 2004. *erm*(C) is the predominant genetic determinant for the expression of resistance to macrolides among methicillin-resistant *Staphylococcus aureus* clinical isolates in Greece. Journal of Antimicrobial Chemotherapy **53**:814-817.

410. **Stephan, R., C. Annemuller, A. A. Hassan, and C. Lammler**. 2001. Characterization of enterotoxigenic *Staphylococcus aureus* strains isolated from bovine mastitis in north-east Switzerland. Veterinary Microbiology **78**:373-382.

411. **Steward, C. D., P. M. Raney, A. K. Morrell, P. P. Williams, L. K. McDougal, L. Jevitt, J. E. McGowan Jr, and F. C. Tenover**. 2005. Testing for induction of clindamycin resistance in erythromycin-resistant isolates of *Staphylococcus aureus*. Journal of Clinical Microbiology **43**:1716-1721.

412. **StGeorge, C., K. E. Russell, and J. B. Wilson**. 1962. Characteristics of staphylococci from bovine milk. Journal of Infectious Diseases **110**:75-79.

413. **Stobberingh, E., A. van den Bogaard, N. London, C. Driessen, J. Top, and R. Willems**. 1999. Enterococci with glycopeptide resistance in turkeys, turkey farmers, turkey slaughterers, and (sub)urban residents in the south of the Netherlands: Evidence for transmission of vancomycin resistance from animals to humans? Antimicrobial Agents and Chemotherapy **43**:2215-2221.

414. **Strommenger, B., C. Kettlitz, T. Weniger, D. Harmsen, A. W. Friedrich, and W. Witte**. 2006. Assignment of staphylococcus Isolates to groups by *spa* typing, SmaI macrorestriction analysis, and multilocus sequence typing. Journal of Clinical Microbiology **44**:2533-2540.

415. **Strommenger, B., C. Kehrenberg, C. Kettlitz, C. Cuny, J. Verspohl, W. Witte, and S. Schwarz**. 2006. Molecular characterization of methicillin-resistant *Staphylococcus aureus* strains from pet animals and their relationship to human isolates. Journal of Antimicrobial Chemotherapy **57**:461-465.

416. **Suller, M. T. E. and A. D. Russell**. 1999. Antibiotic and biocide resistance in methicillin-resistant staphylococcus aureus and vancomycin-resistant enterococcus. Journal of Hospital Infection **43**:281-291.

417. **Suller, M. T. E. and A. D. Russell**. 2000. Triclosan and antibiotic resistance in *Staphylococcus aureus*. Journal of Antimicrobial Chemotherapy **46**:11-18.

418. **Sunde, M. and M. Norstrom**. 2006. The prevalence of, associations between and conjugal transfer of antibiotic resistance genes in *Escherichia coli* isolated from Norwegian meat and meat products. Journal of Antimicrobial Chemotherapy **58**:741-747.

419. **Swaminathan, A., D. Massasso, I. Gotis-Graham, and I. Gosbell**. 2006. Fulminant methicillin-sensitive *Staphylococcus aureus* infection in a healthy adolescent, highlighting 'Panton-Valentine leucocidin syndrome'. Internal Medicine Journal **36**:744-747.

420. Swann committee. Report of the joint committee on the use of antibiotics in animal husbandry and veterinary medicine. 1969. London, Her Majesties Stationary Office.

421. **Takahashi, T., I. Satoh, and N. Kikuchi**. 1999. Phylogenetic relationships of 38 taxa of the genus staphylococcus based on 16S rDNA gene sequence analysis. International Journal of Systematic Bacteriology 49:725-728.

422. **Tejedor Junco, M. T. and J. L. Martin Barrasa**. 2002. Identification and antimicrobial susceptibility of coagulase positive staphylococci isolated from healthy dogs and dogs suffering from otitis externa. Journal of Veterinary Medicine **49**:419-423.

423. **Tenover, F. C., R. D. Arbeit, G. Archer, J. Biddle, S. Byrne, R. V. Goering, G. Hancock, G. A. Herbert, B. Hill, R. Hollis, W. R. Jarvis, B. Kreiswirth, W. Eisner, J. Maslow, L. K. McDougal, J. M. Miller, M. Mulligan, and M. A. Pfaller**. 1994. Comparison of traditional and molecular methods of typing islates of *Staphylococcus aureus*. Journal of Clinical Microbiology **32**:407-415.

424. **Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan**. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed- field gel electrophoresis: criteria for bacterial strain typing. Journal of Clinical Microbiology **33**:2233-2239.

425. **Tenover, F. C., J. W. Biddle, and M. V. Lancaster**. 2001. Increasing resistance to vancomycin and other glycopeptides in *Staphylococcus aureus*. Emerging Infectious Diseases **7**:327-332.

426. **Teuber, M.** 2001. Veterinary use and antibiotic resistance. Current Opinion in Microbiology **4**:493-499.

427. **Thal, L. A. and M. J. Zervos**. 1999. Occurrence and epidemiology of resistance to virginiamycin and streptogramins. Journal of Antimicrobial Chemotherapy **43**:171-176.

428. The Veterinary Medicines Directorate. Sales of Antimicrobial Products used as Veterinary Medicines, Growth Promoters and Coccidiostats in the UK, 2005 Report. 1-35. 2006.

429. **Threlfall, E. J.** 2002. Antimicrobial drug resistance in Salmonella: problems and perspectives in food- and water-borne infections. FEMS Microbiology Reviews **26**:141-148.

430. **Tollefson, L. and W. T. Flynn**. 2002. Impact of antimicrobial resistance on regulatory policies in veterinary medicine: Status report. AAPS PharmSci **4**:1-10.

431. **Tomlin, J., M. J. Pead, D. H. Lloyd, S. Howell, F. Hartmann, H. A. Jackson, and P. Muir**. 1999. Methicillin-resistant *Staphylococcus aureus* infections in 11 dogs. Veterinary Record **144**:60-64.

432. **Torres-Viera, C. and L. M. Dembry**. 2004. Approaches to vancomycin-resistant enterococci. Current Opinion in Infectious Diseases **17**:541-547.

433. **Townsend, D. E., S. Bolton, N. Ashdown, and W. B. Grubb**. 1985. Transfer of plasmid-borne aminoglycoside resistance determinants in staphylococci. Journal of Medical Microbiology **20**:169-185.

434. **Trzcinski, K., B. S. Cooper, W. Hryniewicz, and C. G. Dowson**. 2000. Expression of resistance to tetracyclines in strains of methicillin-resistant *Staphylococcus aureus*. Journal of Antimicrobial Chemotherapy **45**:763-770.

435. **Tsiodras, S., H. S. Gold, G. Sakoulas, G. M. Eliopoulos, C. Wennersten, L. Venkataraman, J. Moellering, and M. J. Ferraro**. 2001. Linezolid resistance in a clinical isolate of *Staphylococcus aureus*. The Lancet **358**:207-208.

436. **Turnidge, J.** 2004. Antibiotic use in animals--prejudices, perceptions and realities. Journal of Antimicrobial Chemotherapy **53**:26-27.

437. **Udo, E. E., N. Al-Sweih, and B. C. Noronha**. 2003. A chromosomal location of the *mup*A gene in *Staphylococcus aureus* expressing high-level mupirocin resistance. Journal of Antimicrobial Chemotherapy **51**:1283.

438. **Udo, E. E. and W. B. Grubb**. 1990. Conjugal transfer of plasmid pWBG637 fron *Staphylococcus aureus* to *Staphylococcus epidermidis* and *Streptococcus faecalis*. FEMS Microbiology Letters **60**:183-187.

439. **Udo, E. E. and W. B. Grubb**. 1996. A phage-mediated transfer of chromosomally integrated tetracycline resistance plasmid in *Staphylococcus aureus*. Current Microbiology **32**:286-290.

440. **Udo, E. E., L. E. Jacob, and E. M. Mokadas**. 1997. Conjugative transfer of high-level mupirocin resistance from *Staphylococcus haemolyticus* to other staphylococci. Antimicrobial Agents and Chemotherapy **41**:693-695.

441. **Uhl, S. and M. Sasatsu**. 1993. Triclosan-resistant *Staphylococcus aureus*. The Lancet **342**:248.

442. **Uzzau, S., D. J. Brown, T. Wallis, S. Rubino, G. Leori, S. Bernard, J. Casadesus, D. J. Platt, and J. E. Olsen**. 2000. Host adapted serotypes of *Salmonella enterica*. Epidemiology and Infection **125**:229-255.

443. **van Belkum, A., D. C. Melles, J. K. Peeters, W. van Leeuwen, E. van Duijkeren, X. W. Huijsdens, E. Spalburg, A. J. de Neeling, and H. Verbrugh**. 2008. Methicillin resistant and susceptible *Staphylococcus aureus* sequence type 398 in pigs and humans. Emerging Infectious Diseases **14**:479-483.

444. **van Belkum, A., J. Kluytmans, W. van Leeuwen, R. Bax, W. Quint, E. Peters, A. Fluit, C. Vandenbroucke-Grauls, A. van den Brule, and H. Koeleman**. 1995. Multicenter evaluation of arbitrarily primed PCR for typing of *Staphylococcus aureus* strains. Journal of Clinical Microbiology **33**:1537-1547.

445. **van den Bogaard, A. E., N. Bruinsma, and E. E. Stobberingh**. 2000. The effect of banning avoparcin on VRE carriage in The Netherlands. Journal of Antimicrobial Chemotherapy **46**:146-148.

446. **van den Bogaard, A. E., N. London, C. Driessen, and E. E. Stobberingh**. 2001. Antibiotic resistance of faecal *Escherichia coli* in poultry, poultry farmers and poultry slaughterers. Journal of Antimicrobial Chemotherapy **47**:763-771.

447. **van den Bogaard, A. E. and E. E. Stobberingh**. 1999. Antibiotic usage in animals: impact on bacterial resistance and public health. Drugs **58**:589-607.

448. **van den Bogaard, A. E., L. B. Jensen, and E. E. Stobberingh**. 1997. Vancomycin-resistant enterococci in turkeys and farmers. New England Journal of Medicine **337**:1558-1559.

449. **van den Bogaard, A. E. and E. E. Stobberingh**. 2000. Epidemiology of resistance to antibiotics: Links between animals and humans. International Journal of Antimicrobial Agents **14**:327-335.

450. **van Leeuwen, W. B.** 2002. Validation of binary typing of *S. aureus* strains. Journal of Clinical Microbiology. **37**:664-674

451. **van Leeuwen, W., W. van Nieuwenhuizen, C. Gijzen, H. Verbrugh, and A. van Belkum**. 2000. Population studies of methicillin-resistant and -sensitive *Staphylococcus aureus* strains reveal a lack of variability in the *agr*D gene, encoding a staphylococcal autoinducer peptide. Journal of Bacteriology **182**:5721-5729.

452. **van Leeuwen, W. B., D. C. Melles, A. Alaidan, M. Al-Ahdal, H. A. M. Boelens, S. V. Snijders, H. Wertheim, E. van Duijkeren, J. K. Peeters, P. J. van der Spek, R. Gorkink, G. Simons, H. A. Verbrugh, and A. van Belkum**. 2005. Host- and tissue-specific pathogenic traits of *Staphylococcus aureus*. Journal of Bacteriology **187**:4584-4591.

453. **van Lunen, T. A.** 2003. Growth performance of pigs fed diets with and without tylosin phosphate supplementation and reared in a biosecure all-in all-out housing system. Canadian Veterinary Journal **44**:571-576.

454. **Vancraeynest, D., F. Haesebrouck, A. Deplano, O. Denis, C. Godard, C. Wildemauwe, and K. Hermans**. 2006. International dissemination of a high virulence rabbit *Staphylococcus aureus* clone. Journal of Veterinary Medicine Series B **53**:418-422.

455. **Vasudevan, P., M. K. M. Nair, T. Annamalai, and K. S. Venkitanarayanan**. 2003. Phenotypic and genotypic characterization of bovine mastitis isolates of *Staphylococcus aureus* for biofilm formation. Veterinary Microbiology **92**:179-185.

456. **Vautor, E., G. Abadie, J.-M. Guibert, C. Huard, and M. Pepen**. 2003. Genotyping of *Staphylococcus aureus* isolated from various sites on farms with diary sheep using pulsed-field gel electrophoresis. Veterinary Microbiology **96**:69-79.

457. **Vautor, E., G. Abadie, J. M. Guibert, N. Chevalier, and M. Pepin**. 2005. Nasal carriage of *Staphylococcus aureus* in dairy sheep. Veterinary Microbiology **106**:235-239.

458. **Vintov, J., Aarestrup F.M., C. E. Zinn, and J. E. Olsen**. 2003. Association between phage types and antimicrobial resistance among bovine *Staphylococcus aureus* from 10 countries. Veterinary Microbiology **95**:133-147.

459. **Vintov, J., F. M. Aarestrup, C. Elsberg Zinn, and J. E. Olsen**. 2003. Phage types and antimicrobial resistance among Danish bovine *Staphylococcus aureus* isolates since the 1950s. Veterinary Microbiology **97**:63-72.

460. **Von Ruden, K. L., V. M. Amin, and N. F. Olson**. 1964. Variability in resistance of coagulase-positive staphylococci to hydrogen peroxide. Journal of Dairy Science **50**:488-491.

461. **Voss, A., F. Loeffen, J. Bakker, C. Klaassen, and M. Wulf**. 2005. Methicillin-resistant *Staphylococcus aureus* in pig farming. Emerging Infectious Diseases **11**:1966.

462. **Waage, S., J. Bjorland, D. A. Caugant, H. Oppegaard, T. Tollerstrud, T. Mork, and F. M. Aarestrup**. 2002. Spread of *Staphylococcus aureus* resistant to penicillin and tetracycline within and between Dairy Herds. Epidemiology and Infection **129**:193-202.

463. **Waldron, D. E. and J. A. Lindsay**. 2006. *Sau*1: a novel lineage-specific type I restriction-modification system that blocks horizontal gene transfer into *Staphylococcus aureus* and between *S. aureus* isolates of different lineages. Journal of Bacteriology **188**:5578-5585.

464. **Walker, E. S., F. Levy, M. Shorman, G. David, J. Abdalla, and F. A. Sarubbi**. 2004. A decline in mupirocin resistance in methicillin-resistant *Staphylococcus aureus* accompanied administrative control of prescriptions. Journal of Clinical Microbiology **42**:2792-2795.

465. **Wallmann, J.** 2006. Monitoring of antimicrobial resistance in pathogenic bacteria from livestock animals. International Journal of Medical Microbiology **296**:81-86.

466. **Weese, J. S., A. Archambault, B. M. Willey, H. Dick, P. Hearns, B. N. Kreiswirth, B. Said-Salim, A. McGeer, Y. Likhoshvay, J. F. Prescott, and D. E. Low**. 2005. Methicillin-resistant *Staphylococcus aurues* in horse and horse personnel, 2000-2002. Emerging Infectious Diseases **11**:430-435.

467. **Weese, J. S., F. Caldwell, B. M. Willey, B. N. Kreiswirth, A. McGeer, J. Rousseau, and D. E. Low**. 2006. An outbreak of methicillin-resistant *Staphylococcus aureus* skin infections resulting from horse to human transmission in a veterinary hospital. Veterinary Microbiology **114**:160-164.

468. **Weese, J. S., H. Dick, B. M. Willey, A. McGeer, B. N. Kreiswirth, B. Innis, and D. E. Low**. 2006. Suspected transmission of methicillin-resistant *Staphylococcus aureus* between domestic pets and humans in veterinary clinics and in the household. Veterinary Microbiology **115**:148-155.

469. **Weese, J. S., J. Rousseau, J. L. Traub-Dargatz, B. M. Willey, A. J. McGeer, and D. E. Low**. 2005. Community-associated methicillin resistant *Staphylococcus aureus* in horses and humans who work with horses. Journal of the American Veterinary Medical Association **226**:580-583.

470. **Wegener, H. C., F. M. Aarestrup, L. B. Jensen, A. M. Hammerum, and F. Bager**. 1999. Use of antimicrobial growth promoters in food animals and *Enterococcus faecium* resistance to therapeutic antimicrobial drugs in Europe. Emerging Infectious Diseases **5**:329-335.

471. **Wegener, H. C., J. L. Watts, S. A. Salmon, and R. J. Yancey, Jr.** 1994. Antimicrobial susceptibility of *Staphylococcus hyicus* isolated from exudative epidermitis in pigs. Journal of Clinical Microbiology **32**:793-795.

472. **Wegener, H. C.** 2003. Antibiotics in animal feed and their role in resistance development. Current Opinion in Microbiology **6**:439-445.

473. **Wei, Y., G. Deikus, B. Powers, V. Shelden, T. A. Krulwich, and D. H. Bechhofer**. 2006. Adaptive gene expression in *Bacillus subtilis* strains deleted for *tet*L. Journal of Bacteriology **188**:7090-7100.

474. **Weisblum, B.** 1995. Erythromycin resistance by ribosome modification. Antimicrobial Agents and Chemotherapy **39**:577-585.

475. **Welton, L. A., L. A. Thal, M. B. Perri, S. Donabedian, J. McMahon, J. W. Chow, and M. J. Zervos**. 1998. Antimicrobial resistance in enterococci Isolated from turkey flocks fed virginiamycin. Antimicrobial Agents and Chemotherapy **42**:705-708.

476. **Werckenthin, C., M. Cardoso, J. L. Martel, and S. Schwarz**. 2001. Antimicrobial resistance in staphylococci from animals with particular reference to bovine *Staphylococcus aureus*, porcine *Staphylococcus hyicus*, and canine *Staphylococcus intermedius*. Veterinary Research **32**:341-362.

477. **Wertheim, H. F., D. C. Melles, M. C. Vos, W. van Leeuwen, A. van Belkum, H. A. Verbrugh, and J. L. Nouwen**. 2005. The role of nasal carriage in *Staphylococcus aureus* infections. The Lancet Infectious Diseases **5**:751-762.

478. **Westh, H., D. M. Hougaard, J. Vuust, and V. T. Rosdahl**. 1995. Prevalence of *erm* gene classes in erythromycin-resistant *Staphylococcus aureus* strains isolated between 1959 and 1988. Antimicrobial Agents and Chemotherapy **39**:369-373.

479. **White, A.** 1963. Increased infection rates in heavy nasal carriers of coagulase positive staphylococci. Antimicrobial Agents and Chemotherapy **161**:667-70.

480. **White, D. G., S. Zhao, R. Sudler, S. Ayers, S. Friedman, S. Chen, P. F. McDermott, S. McDermott, D. D. Wagner, and J. Meng**. 2001. The isolation of antibiotic-resistant salmonella from retail ground meats. New England Journal of Medicine **345**:1147-1154.

481. **Wierup, M.** 2001. The Swedish experience of the 1986 year ban of antimicrobial growth promoters, with special reference to animal health, disease prevention, productivity, and usage of antimicrobials. Microbial Drug Resistance **7**:183-190.

482. **Wierup, M.** 2000. The control of microbial diseases in animals: alternatives to the use of antibiotics. International Journal of Antimicrobial Agents **14**:315-319.

483. **Willems, R. J. L., J. Top, N. van den Braak, A. van Belkum, H. Endtz, D. Mevius, E. Stobberingh, A. van den Bogaard, and J. D. A. van Embden**. 2000. Host specificity of vancomycin-resistant *Enterococcus faecium*. The Journal of Infectious Diseases **182**:816-823.

484. **Winokur, P. L., A. Brueggemann, D. L. DeSalvo, L. Hoffmann, M. D. Apley, E. K. Uhlenhopp, M. A. Pfaller, and G. V. Doern**. 2000. Animal and human multidrug-resistant, cephalosporin-resistant salmonella isolates expressing a plasmid-mediated CMY-2 *Amp*C beta -lactamase. Antimicrobial Agents and Chemotherapy **44**:2777-2783.

485. **Winokur, P. L., D. L. Vonstein, L. J. Hoffman, E. K. Uhlenhopp, and G. V. Doern**. 2001. Evidence for transfer of CMY-2 *Amp*C {beta}-lactamase plasmids between *Escherichia coli* and salmonella isolates from food animals and humans. Antimicrobial Agents and Chemotherapy **45**:2716-2722.

486. **Winslow, C. E. A., W. Rothberg, and E. I. Parsons**. 1920. Journal of Bacteriology 5:145-167.

487. **Witte, W., B. Strommenger, C. Stanek, and C. Cuny**. 2007. Methicillin-resistant *Staphylococcus aureus* ST398 in human and animals in central Europe. Emerging Infectious Diseases **13**:255-258.

488. **Witte, W.** 1998. Medical consequences of antibiotic use in agriculture. Science **279**:996-997.

489. **Witte, W.** 2001. Antibiotic use in animal husbandry and resistance development in human infections. APUA Newsletter **16**:1-6.

490. **Witte, W.** 2001. Selective pressure by antibiotic use in livestock. International Journal of Antimicrobial Agents **16**:19-24.

491. **Wright, J. S., III, K. E. Traber, R. Corrigan, S. A. Benson, J. M. Musser, and R. P. Novick**. 2005. The *agr* Radiation: an early event in the evolution of staphylococci. Journal of Bacteriology **187**:5585-5594.

492. **Wulf, M. and A. Voss**. 2000. MRSA in livestock animals-an epidemic waiting to happen? Clinical Microbiology and Infection. **Epub ahead of print**

493. **Young, H.-K.** 1994. Do nonclinical uses of antibiotics make a difference? Infection Control and Hospital Epidemiology **15**:484-487.

494. **Younis, A., G. Leitner, D. E. Heller, Z. Samra, R. Gadba, G. Lubashevsky, M. Chaffer, N. Yadlin, M. Winkler, and A. Saran**. 2000. Phenotypic characteristics of *Staphylococcus aureus* isolated from bovine mastitis in Israeli dairy herds. Journal of Veterinary Medicine Series B **47**:591-597.

495. **Yugueros, J., A. Temprano, M. Sanchez, J. M. Luengo, and G. Naharro**. 2001. Identification of *Staphylococcus spp.* by PCR-restriction fragment length polymorphism of *gap* gene. Journal of Clinical Microbiology **39**:3693-3695.

496. **Yun, H. J., S. W. Lee, G. M. Yoon, S. Y. Kim, S. Choi, Y. S. Lee, E. C. Choi, and S. Kim**. 2003. Prevalence and mechanisms of low- and high-level mupirocin resistance in staphylococci isolated from a Korean hospital. Journal of Antimicrobial Chemotherapy **51**:619-623.

497. **Zadoks, R. N., W. B. van Leeuwen, D. Kreft, L. K. Fox, H. W. Barkema, Y. H. Schukken, and A. van Belkum**. 2002. Comparison of *Staphylococcus aureus* isolates from bovine and human skin, milking equipment, and bovine milk by phage typing, pulsed-field gel electrophoresis, and binary typing. Journal of Clinical Microbiology **40**:3894-3902.

498. **Zadoks, R., W. van Leeuwen, H. Barkema, O. Sampimon, H. Verbrugh, Y. H. Schukken, and A. van Belkum**. 2000. Application of pulsed-field gel electrophoresis and binary typing as tools in veterinary clinical microbiology and molecular epidemiologic analysis of bovine and human *Staphylococcus aureus* isolates. Journal of Clinical Microbiology **38**:1931-1939.

499. **Zhang, W., B. M. Jayarao, and S. J. Knabel**. 2004. Multi-virulence-locus sequence typing of *Listeria monocytogenes*. Applied and Environmental Microbiology **70**:913-920.

500. **Zhao, S., S. Qaiyumi, S. Friedman, R. Singh, S. L. Foley, D. G. White, P. F. McDermott, T. Donkar, C. Bolin, S. Munro, E. J. Baron, and R. D. Walker**. 2003. Characterization of *Salmonella enterica* serotype Newport isolated from humans and food animals. Journal of Clinical Microbiology **41**:5366-5371.

501. **Zilhao, R., B. Papadopoulou, and P. Courvalin**. 1988. Occurrence of the campylobacter resistance gene *tet*O in *Enterococcus* and *Streptococcus spp.* Antimicrobial Agents and Chemotherapy **32**:1793-1796.