Characterisation of avian isolates of *Staphylococcus aureus*

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Characterisation of avian isolates of

Staphylococcus aureus

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DECLARATION

The thesis is the original work of the author:

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DEDICATION:

This thesis is dedicated to my wife, Zohreh and my daughter, Mahsa and my son, Mohammad, who have provided me with emotional support, patience and encouragement.

It is also dedicated to my mother and in the memory of my father.

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LIST OF ABBREVIATIONS

A	Adenine
agr	Accessory gene regulator
AIPs	Autoinducing peptides
APMSF	4-amidino-phenyl methane-sulfonyl fluoride
AP-PCR	Arbitrarily primed PCR
attB	Integration site
Aur	Aureolysin (metalloprotease)
β Gal	β-galactosidase
BHIB	Brain heart infusion broth
BLAST	Basic local alignment tool
BSA	Bovine serum albumin
C	Cytosine
CAN	Columbia colistin-nalidixic acid
cfu	Colony-forming units
CNS	Coagulase-negative staphylococcal species
CR	Closely related to type 24
CRF	Coagulase-reacting factor
CRP	Coagulase gene restriction profile
CV-agar	Crystal violet agar
DHFR	Dihydrofolate reductase
DNA	Deoxynucleoside triphosphate
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
ERIC	Enterobacterial repetitive intergenic consensus
Eta	Exfoliative toxin A
Etb	Exfoliative toxin B
ETs	Electrophoretic types
E64	(N-(N-(L-3-trans-carboxirane-2-carbonyl)-L-leucyl)-agmatine
G	Guanine
g	Gram
g	Gravity

h	Hour(s)
H_2O	Distilled water
hla	Alpha-haemolysin gene
hlb	Beta-haemolysin gene
hld	Delta-haemolysin gene
hlg	Gamma-haemolysin gene
IR	Inverted repeat
IS	Insertion sequence
kb	Kilobase(s)
kDa	Kilodalton
LB	Luria Bertani
MBSU	Molecular Biology Support Unit
mg	Milligram
µg	Microgram
µl	Microlitre
µM	Micromole
MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequence typing
min	Minutes
ml	Millilitre(s)
MRSA	Methicillin-resistant *S. aureus*
MSSA	Methicillin-sensitive *S. aureus*
MSA	Mannitol salt agar
NA	Nutrient agar
NCBI	National Center for Biotechnology Information
NCTC	National Collection of Type Cultures
NHS	Non-host specific (biotype)
ng	Nanogram
ORF	Open reading frame
°C	Degrees Celsius
PAGE	Polyacrylamide gel electrophoresis
PAL	Alkaline phosphatase
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction

PFGE	Pulsed-field gel electrophoresis
pH	Negative logarithm of hydrogen ion concentration
pmole	Picomole
PR	Possibly related to type 24
PTN	Tween-80
RAPD	Randomly amplified polymorphic DNA
REA	Restriction endonuclease analysis
REP	Repetitive extragenic palindromic element
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
s	Second(s)
sar	Staphylococcal accessory regulator protein
Scp	Cysteine protease
SDS	Sodium dodecyl sulphate
SEA	Enterotoxin A
SEB	Enterotoxin B
SEC	Enterotoxin C
SED	Enterotoxin D
SEE	Enterotoxin E
SEG	Enterotoxin G
SEH	Enterotoxin H
SEI	Enterotoxin I
SET-RPLA	Staphylococcal enterotoxin reversed passive latex agglutination
spa	Protein A gene
SspA	Staphylococcal serine protease (V8 protease)
SspB	Second cysteine protease
T	Thymine
TCA	Trichloroacetic acid
Tn	Transposon
TSA	Tryptone soya agar
TSST-1	Toxic shock syndrome toxin-1
U	Unit

UR	Unrelated to type 24
UV	Ultraviolet
V	Volts
v/v	Volume/volume ratio
w/v	Weight/volume ratio

ABSTRACT

This work has involved the characterisation of a number of avian isolates of *S. aureus* from Scotland, England, N. Ireland, America and Sri Lanka. Other avian, human and animal coagulase-positive and coagulase-negative species served as control organisms in the various test procedures. The isolates were mainly from clinically-diseased birds. More than half of the avian isolates of *S. aureus* were from N. Ireland. The identity of the strains was confirmed by conventional cultural and biochemical tests (including Rapidec Staph and API Staph) and by rapid molecular methods. The strains were also characterised in terms of their plasmid profiles and antibiotic resistance. A high proportion (95%) of the *S. aureus* isolates contained one or more plasmids ranging in size from 1.6 to 16 kb. The sensitivity of these strains to a number of antibiotics was determined and 96% of the avian *S. aureus* isolates were resistant to one or more of the antibiotics tested. A high proportion (71%) of isolates were resistant to tetracycline, kanamycin, tobramycin and gentamicin. A close correlation between tetracycline resistance and the presence of a 4.4 kb plasmid was demonstration by eletrotransformation of the plasmid into tetracycline sensitive strains.

The main part of the work was to develop PCR techniques for identification and typing of avian *S. aureus*. Different primer sets were applied in order to generate reproducible profiles of the PCR amplimers. Of the 86 avian *S. aureus* studied, 12, 7, 4 and 5 groups were recognised by the use of ribosomal, REP, coagulase gene and protein A gene primers respectively. PCR-ribotyping (method 1) produced a relatively simple pattern which was useful for rapid identification of these isolates and for differentiation of these isolates from each other. These PCR fingerprinting methods were simple to perform and reproducible. The majority (80%) of the strains even from different geographical locations had similar "typical avian" profiles whereas 20% had atypical avian profiles.

Results showed that many of the virulence factors characterised in this study in avian *S. aureus* were likely to be regulated by the *agr* and *sar* loci. Sequencing of the *agrD* region in selected strains suggested that 80% of avian (typical

avian) isolates belonged to *agr* specificity group II and the other (20%) (atypical avian) strains belonged to *agr* specificity groups I or III.

An investigation by multiplex PCR for eight toxin genes (encoding enterotoxins A-E, exfoliative toxins A-B and toxic shock syndrome toxin-1) revealed that only 5% of the avian isolates produced one or more of these toxin genes and all were atypical by other criteria. A multiplex PCR developed to detect haemolysin (α, γ and δ) genes showed that all the 86 avian *S. aureus* isolates had the α and δ-haemolysin genes and a majority (86%) of the strains also had the γ-haemolysin gene. Many of those that lacked the γ-haemolysin gene were atypical by other criteria. Only 5% of the avian *S. aureus* strains possessed the β-haemolysin gene and, again, these isolates were atypical by other criteria.

One of the most striking findings in the present study was that a single dominant protein band of 22 kDa was present in the culture supernates of 89.5% of avian *S. aureus* strains. N-terminal sequence analysis of the 22 kDa protein gave a sequence that was almost identical to that previously reported for a thiol (cysteine) protease in an avian *S. aureus* strain and the presence of a cysteine protease gene in the avian strains was confirmed by PCR and sequence analysis of the amplimer. Other tests with different inhibitors and an activator of protease activity supported the conclusion that the 22 kDa band represented a cysteine protease.

1. INTRODUCTION

1.1 STAPHYLOCOCCI

1.1.1 History and bacteriological characteristics

Ogston (1882, cited by Baird-Parker, 1972) introduced the name "*Staphylococcus*" (from *staphylé*, a bunch of grapes) for the group of micrococci causing inflammation and suppuration. Rosenbach (1884, cited by Baird-Parker, 1972) used the term in a taxonomic sense and provided the first description of the genus *Staphylococcus*. Members of the genus *Staphylococcus* are Gram-positive cocci (0.5-1.5 μm) that occur singly, in pairs, tetrads, short chain, and irregular "grape-like" clusters. They are non-motile and non-spore forming. Most species demonstrate catalase activity. Most grow in the presence of 10% sodium chloride and at 18-40 °C. They are usually unencapsulated or have limited capsule formation. Most species are facultative anaerobes and except for *S. saccharolyticus* and *S. aureus* subsp. *anaerobius*, their growth is more rapid and abundant under aerobic conditions. The G+C content of staphylococci was found to be 30-40 mol% (Kocur *et al.,* 1971). The cell walls of staphylococci contain both peptidoglycan and teichoic acid and the peptidoglycan contains large amount of glycine (Schleifer and Kandler, 1972).

Species: There are 32 species currently recognized in the genus *Staphylococcus* (Kloos, 1998). Coagulase-positive species of *Staphylococcus* include *S. aureus* subsp. *aureus*, *S. aureus* subsp. *anaerobius*, *S. hyicus, S. intermedius*, *S. delphini*, and *S. schleiferi* subsp. *coagulase* (Pantuceck *et al.,* 1996).

Staphylococcus species can be identified on the basis of colony morphology, coagulase production, oxygen requirements, haemolysins, resistance to certain antibiotics, various enzyme activities and acid production from certain carbohydrates under aerobic conditions. Most clinically significant coagulase-positive species are compared in **Table 1.1**.

Table 1.1 Differentiation of coagulase-positive *Staphylococcus* species (Data from Kloos and Lambe 1991, Kloos, 1998).

Property[a]	*S. aureus*	*S. schleiferi*	*S. intermedius*	*S. delphini*	*S. hyicus*
Colony size (large)[b]	+	-	+	+	+
Colony pigment[c]	+	-	-	-	-
Anaerobic growth[d]	+	+	(+)	(+)	+
Aerobic growth	+	+	+	+	+
Staphylocoagulase[e]	+	±	+	+	d
Clumping factor[f]	+	±	d	-	-
Heat-stable nuclease	+	+	+	-	+
Haemolysins	+	(+)	d	+	-
Catalase	+	+	+	+	+
Oxidase	-	-	-	-	-
Alkaline phosphatase	+	+	+	+	+
Arginine arylamidase	-	-	-	ND	-
Pyrrolidonyl arylamidase	-	+	+	ND	-
Ornithine decarboxylase	-	-	-	ND	-
Urease	d	-	+	+	d
β-glucosidase	+	-	d	ND	d
β-glucuronidase	-	-	-	ND	+
β-galactosidase	-	(+)	+	ND	-
Arginine utilization	+	+	d	+	+
Acetoin production	+	+	-	-	-
Nitrate reduction	+	+	+	+	+
Esculin hydrolysis	-	-	-	ND	-
Novobiocin resistance	-	-	-	-	-
Polymyxin B resistance	+	-	-	ND	+
D-trehalose	+	d	+	-	+
D-mannitol	+	-	(d)	(+)	-
D-mannose	+	+	+	+	+
D-turanose	+	-	d	ND	-
D-xylose	-	-	-	-	-
D-cellobiose	-	-	-	ND	-
L-arabinose	-	-	-	-	-
Maltose	+	-	(±)	+	-
α-lactose	+	-	d	+	+
Sucrose	+	-	+	+	+
N-acetylglucosamine	+	(+)	+	ND	+
Raffinose	-	-	-	ND	-

[a] Symbols (unless otherwise indicated): +, 90% or more strains positive; ±, 90% or more strains weakly positive; -, 90% or more strains negative; d, 11 to 89% of strains positive; ND, not determined; ( ), delayed reaction.

[b] Positive is defined as a colony diameter of ≥6 mm after incubation on P agar at 34 to 35°C for 3 days and at room temperature (ca. 25 °C) for an additional 2 days (Kloos and Schleifer, 1975a).

[c] Positive is defined as the visual detection of carotenoid pigments (e.g., yellow, yellow-orange, or orange) during colony development at normal incubation or room temperatures. Pigments may be enhanced by the addition of milk, fat, glycerol monoacetate, or soaps to P agar.

[d] Anaerobic growth in a semisolid thioglycolate medium (Kloos and Schleifer, 1975a). Symbols: +, moderate or heavy growth down tube within 18 to 24; ±, heavier growth in the upper portion and weaker growth in the lower, anaerobic portion of tube; -, no visible growth within 48 h, but by 72 to 96 h very weak diffuse growth or a few scattered, small colonies may be observed in the lower portion of tube; ( ), delayed growth appearing within 24 to 72 h, sometimes noted as large discrete colonies in the lower portion of tube.

[e] ±, staphylocoagulase test for *S. schlieferi* subsp. *schleiferi* was negative, but for *S. schlieferi* subsp. *coagulase* was positive.

[f] ±, clumping factor test for *S. schlieferi* subsp. *schleiferi* was positive, but for *S. schlieferi* subsp. *coagulase* was negative

Avian species: Staphylococci isolated frequently from poultry include *S. aureus* and *S. epidermidis* (Lukasova *et al.,* 1986; Jose *et al.,* 1994). Another species, *S. gallinarum,* has been isolated from processed poultry (Deveriese *et al.,* 1983). *S. hyicus* has been associated with fibrinoheterophilic blepharitis in chickens and turkeys (Cheville *et al.,* 1988), and was isolated in profuse and often pure culture from skin lesions, and from internal organs of chickens and turkeys suffering from pox (Devriese *et al.,* 1992). It was also isolated from five of nine tibiotarsal growth plates of turkeys with stifle joint osteoarthritis (Tate *et al.,* 1993). *S. intermedius* is found frequently on carnivora (Devriese *et al.,* 1984) and has been isolated also from certain other mammals and birds (Kloos, 1998). *S. arlettae* has been isolated from poultry and goats (Schleifer *et al.,* 1984).

Cutaneous habitats: Staphylococci are widespread in nature, though they are mainly found living on the skin, skin glands, and mucous membranes of mammals and birds (Noble and Somerville 1974; Devriese *et al.,* 1978; Devriese *et al.,* 1983; Akatov *et al.,* 1985; Devriese *et al.,* 1986; Kloos *et al.,* 1991).

Cutaneous habitats in birds: *Staphylococcus* spp. are ubiquitous, normal inhabitants of skin and mucous membranes, and are common organisms in environments where poultry are hatched, reared or processed. Most staphylococcal species are considered to be normal flora, which help suppress other possible pathogens by their presence through interference or competitive exclusion. Some have the potential to be pathogenic and produce disease if allowed entry through the skin or mucous membranes (Skeeles, 1997).

Staphylococci and *S. aureus* in particular were found to be present on the body surfaces of live poultry (Devriese *et al.,* 1975). *S. aureus* has also been isolated from the skin and nasopharynx of poultry and of wild birds (Cooper and Needham, 1976).

Other habitats: Staphylococci have been detected in the pharynx, conjuctiva, mouth, blood, mammary glands, faeces, bodily discharges, excretions and intestinal, genitourinary and respiratory tracts of their hosts (Elek, 1959). Small numbers of staphylococci have been found in air, dust, soil and water, and on inanimate surfaces or fomites, molluscs, insects and plants in areas frequented by mammals and birds (Elek, 1959; Kloos and Schleifer, 1981).

1.1.2 Culture and isolation

1.1.2.1 Colonial appearance

Staphylococci produce distinctive colonies on a variety of commercial, selective and non-selective agar media. The commonly used selective media include mannitol-salt agar, lipase-salt-mannitol agar, phenylethylalcohol agar; Columbia colistin-nalidixic acid (CAN) agar, and Baird-Parker agar base supplemented with egg yolk tellurite enrichment. These media inhibit the growth of Gram-negative bacteria, but allow the growth of staphylococci and certain other Gram-positive bacteria. Baird-Parker agar base supplemented with egg yolk tellurite enrichment is widely recommended for the detection and enumeration of *S. aureus* in foods (Schwab *et al.,* 1984). This medium and the other selective media were designed to distinguish *S. aureus* from other species on the basis of colonial and biochemical properties; however, with the discovery of additional staphylococcal species that share some properties with *S. aureus*, it is now recognised that such a distinction can not be made accurately. Schleifer-Krämer agar (Schleifer and Krämer, 1980) is used by some laboratories for the selective isolation and enumeration of staphylococci from foods and other heavily contaminated sources. Incubation of cultures on selective media should be for at least 48-72 h at 35-37 °C for colony development.

Staphylococci from a variety of clinical specimens are usually isolated in primary culture on blood agar (e.g. tryptic soy agar supplemented with 5% sheep blood), following an incubation period of 18-24 h at 35-37 °C. In this short time,

most staphylococcal species will produce abundant growth and well isolated colonies will be 1-3 mm in diameter, circular, smooth and raised, with a butyrous consistency; unfortunately, however colonies of most of the species and subspecies can not be distinguished from one another. Considering the necessity for the rapid identification of *S. aureus* in clinical specimens, it is fortunate that colonies of pigmented haemolytic strains of *S. aureus* can be tentatively identified within 24 h. Suggestions to extend the incubation period, to allow for the development of species, subspecies, and strain-specific colonial features, have been largely ignored, until recently. Now there is an appreciation that certain coagulase-negative staphylococcal species (CNS) are opportunistic pathogens and that individual species, subspecies and strains should be identified in multiple specimens taken from the site(s) of infection when determining the aetiological agent.

The identification of staphylococci should start with the characterisation of colony morphology (Kloos, 1998). P-agar (Kloos *et al.,* 1974) and tryptic soy agar have been widely used for isolation from the primary cutaneous habitats (Kloos *et al.,* 1991). Colonies of each of the recognised species and subspecies can be distinguished from one another on these media with considerable accuracy, provided reference strains of each of the taxa are included for comparison. Detailed descriptions of colonies have been reported in the original taxonomic descriptions of the species and subspecies. Pigmentation and pigment sectoring patterns are usually more pronounced in colonies growing on these media than on blood agar. When pigment is present, colonies are usually different shades of cream-yellow, yellow, yellow-orange, or orange, depending on the particular species and strain. Some strains of *S. epidermidis*, *S. intermedius* and *S. lentus* demonstrate a subtle violet, pinkish, or brownish pigment. All staphylococcal species grow well on tryptic soy agar, but *S. vitulus*, *S. lentus*, *S. caseolyticus* and some strains of *S. sciuri* grow poorly on P-agar. These species should be cultured on tryptic soy agar or tryptic soy agar supplemented with blood. The anaerobic species *S. saccharolyticus* and *S.*

aureus subsp. *anaerobius* grow well on the above non-selective media under anaerobic conditions, but grow very poorly under aerobic conditions. Brain-heart infusion agar and nutrient agar support good growth of staphylococci, although not many species comparisons have been made of colonies growing on these media.

1.1.2.2 Growth in broth and semi-solid media

Staphylococci grow well in a variety of commercial broth media, including tryptic soy broth, brain-heart infusion broth, nutrient broth and tryptose phosphate broth, with or without the addition of blood. Most of the species are butyrous and easy to emulsify, but the *S. sciuri* species group, *S. caseolyticus* and the strong slime-producing strains of *S. epidermidis* are glutinous and difficult to emulsify. Many of the staphylococcal species can produce abundant anaerobic growth in a semi-solid Brewer's thioglycollate medium within 24-72 h at 35-37 °C (Kloos and Schleifer, 1975b; Kloos and Bannerman, 1995). Species such as *S. aureus*, *S. epidermidis*, *S. lugdunensis* and *S. schleiferi*, which are among the most pathogenic species, produce abundant anaerobic growth with overnight incubation. Some species such as *S. hominis*, *S. auricularis*, *S. kloosii*, *S. equorum*, *S. arlettae*, *S. caseolyticus*, *S. vitulus* and *S. lentus* fail to grow or grow only very slowly in the anaerobic portion of this medium (Kloos, 1998).

1.1.3 Identification of species with commercial biochemical test systems

To expedite the process of identifying staphylococcal species and subspecies in the clinical laboratory, several manufacturers have developed rapid identification kits and automated systems requiring only a few hours to one day for completing tests. With these products, identification of most species and subspecies can be made with an accuracy of 70->90% (Kloos and Wolfshohl, 1982; Crouh *et al.*, 1987; Kloos and George, 1991; Bannerman *et al.*, 1993). Currently, *S. aureus*, *S. epidermidis*, *S. haemolyticus*, *S. capitis* subsp. *capitis*, *S. saprophyticus*, *S. cohnii* subsp. *cohnii*, *S. simulans*, *S. intermedius* and *S. sciuri* can be identified reliably by most of the commercial systems available. Some additional testing may be

required to increase the accuracy of identification above 90%. The rapid identification systems now available include the following: API-STAPH-IDENT, STAPH Trac System, API STAPH, RAPIDEC STAPH and ID 32 STAPH kits and the fully automated Vitek and Vitek, Jr systems, which utilize a Gram Positive Identification (GPI) Card (bioMérieux Vitek Inc., Hazelwood, MO, USA); MicroScan pos ID panel, MicroScan Rapid Pos ID panel, and the Pos Combo and Rapid Pos Combo panels, which combine species identification and antimicrobial susceptibility tests (Dade MicroScan Inc., West Sacramento Microbiology Systems); Sceptor Staphylococcus MIC/ID panel and Sceptor Gram Positive Breakpoint/ID Panel (Becton Dickinson Instrument Systems, Towson, MD, USA); GP MicroPlate test panel (Biology, Haywood, CA, USA); and the Microbial Identification System (MIS) that automates identification by combining cellular fatty acid analysis with computerised high-resolution gas chromatography (MIDI, Newark, DE, USA).

Rapid identification of the species *S. aureus* can be made using the AccuProbe culture identification test for *S. aureus* (Gen-Probe Inc., San Diego, CA, USA). The test is a DNA probe assay directed against rRNA and it is very accurate. This species can also be identified by a new immunoenzymatic assay based on a monoclonal antibody prepared against the *S. aureus* endo-β-N-acetylglucosaminidase (Guardati *et al.,* 1993). These species tests are specially useful in identifying coagulase-negative, protein A-negative, and/or clumping factor-negative mutants of *S. aureus*, that would otherwise be misidentified by coagulase and latex agglutination tests.

1.1.4 Antimicrobial sensitivity

Staphylococci have a record of developing resistance quickly and successfully to antibiotics. Penicillin-resistant *S. aureus* strains began emerging shortly after the introduction of penicillin in medicine in the early 1940s. Today the percentage of penicillin-resistant strains has risen to 75-95% with the highest percentages found among hospital strains (Kloos, 1998). The methicillin-resistant strains

isolated in Britain came from hospitalized patients, were multiply antibiotic-resistant, belong to phage group III, and their resistance to methicillin was heterogenous, affecting only a minority of the cell population. After the mid-1970s, large outbreaks of infection by methicillin-resistant *S. aureus* (MRSA) were recorded in many hospitals in some countries (Schaefler *et al.,* 1981; Pavillard *et al.,* 1982). Many of these outbreaks appear to have been caused by a single epidemic strain that was transferred between hospitals by the movement of patients (Duckworth *et al.,* 1988). Genetic analyses and molecular typing methods have indicated that various isolates of MRSA may have had a clonal origin, and suggest that the methicillin-resistance determinant was acquired by *S. aureus* at some point in time before or around the time of the introduction of methicillin (Kreiswirth *et al.,* 1993). Although the mechanism of this type of resistance is not fully understood, it at least partly involves the presence of modified penicillin-binding proteins with decreased β-lactam affinity (Brakstad and Maeland, 1997).

Erythromycin-resistant staphylococci often have cross-resistance to macrolides (erythromycin, oleandomycin, spiramycin, clarithromycin, azithromycin), lincosamides (lincomycin, clindamycin) and streptogramin type B antibiotics. These antibiotics, collectively designated Macrolids, Lincosamide, and Streptogramins B (MLS), bind to the 50S ribosomal subunit at overlapping binding sites, and binding interferes with transpeptidation and translocation reactions needed for peptide chain elongation. In resistant strains these sites are protected by the N^6, N^6-dimethylation of an adenine-2058 residue at the peptidyl transferase centre of 23S rRNA, a reaction performed by a rRNA methylase. Three distinct rRNA methylase genes have been detected in staphylococci: *ermA, ermB* and *ermC* (Weisblum, 1985a).

Tetracycline resistance is widespread among staphylococcal species and ranks along with β-lactam and MLS resistance as one of the most frequent types of antibiotic resistance found in natural populations of staphylococci (Schwarz *et*

al., 1994). There are two mechanisms of tetracycline resistance recognised in staphylococci. The most common one involves an energy-dependent pumping (efflux) of tetracycline and doxycycline from the cell, so that levels of these antibiotics are reduced below that required to inhibit the ribosome. The efflux protein is most often encoded by the inducible gene *tetK* which is located on class I plasmids of the PT181 family (see section 1.3.2). The second mechanism, one that is controlled by the gene *tetM,* involves ribosome protection such that protein synthesis is unaffected by the presence of tetracycline, doxycycline, or minocycline (Schaefler *et al.,* 1976).

Trimethoprim resistance is either mediated by alterations in the expression of the intrinsic chromosomal *dfr* gene (e.g. *dfrB* of *S. aureus* or *dfrC* of *S. epidermidis*), possibly resulting in overproduction of the native dihydrofolate reductase (DHFR) or a reduced affinity of the native DHFR for trimethoprim, or by the acquisition of a second chromosomal or plasmid *dfr* gene (e.g. *dfrA* in *S. aureus, S. epidermidis* and *S. hominis*) that encodes a trimethoprim-resistant DHFR capable of rescuing the reduction step leading to tetrahydrofolate in the presence of trimethoprim (Galetto *et al.,* 1987; Rouch *et al.,* 1989; Dale *et al.,* 1995).

1.1.4.1 Resistance to antimicrobial agents in avian strains

In a study of the putative transfer of antibiotic resistance from poultry to humans, hens and eggs were examined for the presence of various pathogens. Sensitivity tests showed the presence of strains of *S. aureus* resistant to one or more of penicillin-G, tetracycline, erythromycin, clindamycin, cephalosporins, oxacillin, gentamicin, chloramphenicol, tobramycin, ciporofloxacin, sulphamethoxazole and novobiocin (Papadopoulou *et al.,* 1997; Nawaz *et al.,* 1999; Aarestrup *et al.,* 2000). Methicillin-resistant coagulase-negative staphylococci were isolated from the nares and skin of 1 to 8 week old healthy chickens (Kawano *et al.,* 1996). There is no report about avian MRSA strains.

1.2 CELLULAR COMPONENTS

1.2.1 Cell surface components

The principal structures of the cell envelope or surface of staphylococci are the cell membrane, cell wall and exocellular material. Many of the components of these structures have biological activities and play a vital role in the interaction of staphylococci with their habitat or environment. The cell surface is a mosaic in which the cell wall components and certain membrane components may be exposed sufficiently to interact with external factors. Some of these components may be submerged beneath the surface in staphylococci that produce large amounts of exocellular material.

1.2.1.1 Cell membrane

The cell membrane appears to be a typical lipid-protein bilayer, being composed mainly of phospholipids and proteins. It represents a selective barrier and fulfils many functions, including electron transport, active transport, participation in septum formation, and segregation of DNA. The cytochromes and menaquinones bound to cell membranes are important components of the electron transport system. Phospholipids, glycolipids, menaquinones and carotenoids make up the major lipid components of the membrane. The major polar lipids are phospholipids and glycolipids (Shaw, 1975; Nahaie *et al.,* 1984). The major phospholipids are phosphatidylglycerol, diphosphatidylglycerol (cardiolipin) and phosphatidic acid. Lysylphosphatidylglucose is also a major component of the cell membrane of *S. aureus* and *S. intermedius*. The glycolipids are monoglucosyl and β-diglucosyl diglycerides and phosphatidyl glucose. The (1→6)-β-linked diglucosyl (gentiobiosyl) diacyglycerol of staphylococci is also found in the membrane of *Bacillus* species. There is no information specifically about the cell membrane composition of avian isolates of staphylococci.

1.2.1.2 Cell wall

Peptidoglycan and teichoic acid are the major components of the staphylococcal cell wall. The peptidoglycan amounts to about 50-60% of the dry weight (Schleifer and Kandler, 1972; Schleifer, 1983). The cell wall teichoic acid amounts to about 30-50% of the dry weight, and it is covalently linked to peptidoglycan. The peptidoglycan is the main structural polymer in the wall and it plays an important role in maintaining the spherical shape of the cell. It is a heteropolymer consisting of glycan chains that are cross-linked by short peptides. The glycan moiety is made up of alternating β-1,4-linked units of N-acetylglucosamine and N-acetylmuramic acid. Some of the C-6 hydroxl groups of the muramyl-6-phosphate residue represent the attachment points between peptidoglycan and teichoic acid (Kojima *et al.,* 1985). There is no information specifically about the cell wall composition of avian isolates of staphylococci.

1.2.1.3 Protein A

Protein A is a cell wall-associated protein of *S. aureus*, and it has the ability to bind to the Fc portion of IgG antibodies (Forsgren and Sjöquist, 1966; Forsgren, *et al.,* 1983). Protein A (SpA) of *S. aureus* has been detected in 90-99% of biotype A strains isolated from man (Hájek and Maršálek, 1976; Forsgren *et al.,* 1983). It is detected less frequently in *S. aureus* biotype B strains (2-5%), isolated from pigs and poultry and in biotype C strains (6-40%), isolated from cattle, pigs, sheep and goats. *S. hyicus* produces a type of SpA that is different from that *S. aureus* (Muller *et al.,* 1981). About 85% of *S. intermedius* strains produce an extracellular type of SpA, whereas 4% of strains produce a cell-bound form (Cox *et al.,* 1986). The absence of protein A differentiates *S. aureus* of the poultry biotype from *S. aureus* of the slaughterhouse biotype as described by Isigidi *et al.* (1990).

1.2.1.4 Cell surface adhesins

Staphylococci have a group of cell surface proteins that act as adhesins (microbial surface components recognising adhesive matrix molecules,

MSCRAMMs) and bind to extracellular matrix proteins such as fibronectin, vitronectin, fibrinogen, laminin, bone sialoprotein, thrombospondin, elastin and collagen types I, II and IV, of their host (Wadström and Rozgonyi, 1986; Rydén *et al.,* 1983; 1989; 1990; Switalski *et al.,* 1989; Wadström, 1991).

The matrix proteins play important roles in the systematic organisation of host tissues (e.g. connective tissue, bone tissue) and maintenance of homoeostasis. The two fibronectin-binding adhesins (*FnBPs*) of *S. aureus* are encoded by two distinct, but highly homologous, genes *fnbA* and *fnbB* (Jönsson *et al.,* 1991). *S. aureus* produces a collagen-binding adhesin that is encoded by the gene *cna* (Patti *et al.,* 1992).

The cell surface fibrinogen-binding adhesin (clumping factor) of *S. aureus* is encoded by gene *clfA* (McDevitt *et al.,* 1995). Staphylocoagulase (coagulase) is mainly an extracellular protein of *S. aureus* and certain other species that binds prothrombin and activates the coagulation pathway (Hemker *et al.,* 1975). However, a small fraction of the coagulase molecules remains bound to the cell wall and this fraction can bind fibrinogen as well as prothrombin (Bodén and Flock, 1989). There is no information about cell-surface adhesins in avian isolates of *S. aureus*.

1.2.1.5 Exocellular material

Staphylococcal capsules are located external to the cell wall and they are composed primarily of antigenic (capsular) polysaccharides (Sutherland, 1977). The capsular polysaccharides of *S. aureus* are made up of different combinations of either amino uronic acids (N-acetyl-D-aminoglucuronic acid, N-acetyl-D-aminogalacturonic acid or N-acetyl-D-aminomannuronic acid) or non-amidated uronic acids (glucuronic acid or galacturonic acid) and N-acetyl-D-fucosamine or galactose, depending upon the particular strain (Wilkinson, 1983).

Slime is a complex extracellular substance produced in varying amounts by many, if not most, staphylococci. The amounts of slime produced by a particular strain may vary widely according to genetic factors and growth conditions. Many strains of *S. epidermidis* and some strains of *S. capitis* subsp. *ureolyticus* produce copious amounts of this substance (Peters *et al.,* 1987; Kloos *et al.,* 1992). There is no information about exocellular material produced by avian isolates of *S. aureus*.

1.2.2 Extracellular proteins

S. aureus produces a wide variety of exoproteins that contribute to its ability to colonize and cause disease in mammalian hosts. Nearly all strains are reported to secrete a group of enzymes and cytotoxins which includes four haemolysins (alpha, beta, gamma, and delta), nuclease, proteases, lipases, hyaluronidase, and collagenase. The main function of these proteins may be to convert local host tissues into nutrients required for bacterial growth. Some strains produce one or more additional exoproteins, which include toxic shock syndrome toxin-1 (TSST-1), the staphylococcal enterotoxins (SEA, SEB, SEC1-3, SED, SEE, SEG, SEH, and SEI), the exfoliative toxins (ETA and ETB), and leukocidin. Each of these toxins is known to have potent effects on cells of the host immune system, but many of them have other biological effects as well. Their primary function in vivo may be to inhibit host immune responses to *S. aureus*. TSST-1 and the staphylococcal enterotoxins are also known as pyrogenic toxin superantigens (PTSAgs). Two former names for TSST-1 were staphylococcal pyrogenic exotoxin C and staphylococcal enterotoxin F (Dinges *et al.,* 2000). The PTSAgs are a group of exotoxins secreted by either *S. aureus* or *Streptococcus pyogenes* that have been grouped together because they share several important biological characteristics (Bohach *et al.,* 1990; 1997). The family of PTSAgs presently includes TSST-1, most of the staphylococcal enterotoxins (SEA, SEB, SECn, SED, SEE, and SEH), and the streptococcal pyrogenic exotoxins (SPE A, B, C, F, G, H and J and streptococcal superantigen) (Bergdoll *et al.,* 1973; Bergdoll, 1983; Bohach *et al.,* 1990; 1997).

Each of these exotoxins exhibits at least three biological properties: pyrogenicity; superantigenicity, which refers to the ability of these exotoxins to stimulate proliferation of T-lymphocytes without regard for the antigen specificity of these cells; and the capacity to enhance the lethality of endotoxin in rabbits up to 100,000-fold (Bohach *et al.,* 1990; 1997).

1.2.2.1 Staphylococcal enterotoxins

Eight major toxins have been identified, two very recently (SEG and SEI), and various degrees of investigation into their structures and functions have been undertaken. The SEs, SEA to SEI except SEF, are produced by various coagulase-positive staphylococci. All toxins thus far identified share a number of important properties (Bergdoll *et al.,* 1973; Bergdoll, 1983; Bohach *et al.,* 1990; 1997), including (i) an ability to cause emesis and gastroenteritis in a primate model, (ii) superantigenicity, (iii) intermediate resistance to heat and pepsin digestion, and (iv) tertiary structural similarity (where known) including an intramolecular disulphide bond. SEC can be further divided into three major antigenic subtypes, SEC1, SEC2 and SEC3.

Genigeorgis and Sadler (1966) have emphasized the public health significance of *S. aureus* (ca. 40% of which were found to be enterotoxigenic) strains which could be isolated from the livers of poultry slaughtered commercially. The presence of enterotoxigenic strains of *S. aureus* in foods was suggested as a potential public health hazard because of the ability of these strains to produce intoxication or food poisoning.

In a study of enterotoxin production in poultry strains by Harvey *et al.* (1982), *S. aureus* strains were isolated from end-of-lay poultry carcasses obtained from a plant at two different stages of processing before and after storage at different temperatures. These strains were supplemented with *S. aureus* strains isolated from poultry from a wide range of sources and biotyped, phage-typed, and tested for production of enterotoxins A-E. The isolates were found to consist of poultry

and human specific strains and each of these groups contained strains able to produce enterotoxins. Poultry strains produced only enterotoxin D whereas human strains produced enerotoxins A, C and D. The hen carcasses used in storage experiments were found to be naturally contaminated with enterotoxin D-producing staphylococci. However enterotoxin D could not be detected on any of the carcasses even after storage at temperatures which allowed multiplication of the organisms to occur. Other investigators have suggested that enterotoxin-producing *S. aureus* strains, which can be isolated from poultry carcasses at processing, have their origin from either contaminated equipment or people in the processing plant (Notermans *et al.,* 1982; Adams and Meat, 1983; Thompson and Patterson, 1983).

1.2.2.2 Toxic shock syndrome toxin-1 (TSST-1)

Toxic shock syndrome is an acute and potentially fatal human illness that is characterised by a high fever, diffuse erythematous rash, desquamation of the skin 1 to 2 weeks after onset (if not fatal before this time), hypotension, and involvement of three or more organ systems (Todd *et al.,* 1978; Shands *et al.,* 1980; Davis *et al.,* 1980; Reingold *et al.,* 1982; Chesney, 1989; Deresiewicz, 1990). The illness was initially brought to the attention of the medical community in 1978 by Todd *et al.* (1978), who recognised it as a major systemic illness associated with non-invasive *S. aureus* infections in children. The illness was designated TSS by these investigators. In the early 1980s, an epidemic of TSS occurred among young women in the United States. Nearly all of these cases of TSS were associated with menstruation, the use of tampons (particularly those of higher absorbency), and the presence of *S. aureus* localised to cervical or vaginal colonization (Davis *et al.,* 1980; Shands *et al.,* 1980). The absence of detectable bacteremia in these patients suggested that TSS resulted from intoxication with products elaborated by *S. aureus*. TSST-1 was the first marker toxin identified for TSS (Bergdoll *et al.,* 1981; Bergdoll and Schlievert 1984), and this toxin is currently accepted as the cause of 100% of menstruation-associated TSS cases. TSST-1 is the only PTSAg known to cause TSS from

intravaginal sources; this is presumably due to its unique capacity to cross mucosal surfaces. Of the *S. aureus* strains isolated in TSS cases not associated with tampon usage, approximately 50% produce TSST-1, 47% produce SEB, and 3% produce SEC (Schlievert, 1986; Bohach *et al.,* 1990). Coagulase-negative staphylococci have not been shown to cause TSS. There is no information about TSST-1 produced by avian isolates.

1.2.2.3 Exfoliative toxins

Like the enterotoxins, the exfoliative toxins have been purified to relative homogeneity and, in pure form, have been shown to cause effects in animals similar to what is observed in infected patients. The purified exfoliative toxins, also called epidermolytic toxins, have produced skin symptoms of staphylococcal scalded skin syndrome (SSSS), a disease most often associated with neonates (Gemmell, 1995). This disease starts with a localised skin lesion, followed by widespread erythema and exfoliation. The name of the disease comes from the fact that the skin looks as though it has been burned. Epidemiologically, exfoliative toxin-producing strains are correlated with staphylococcal bullous impetigo (Piemont *et al.,* 1984; Murono *et al.,* 1988). The genes for exfoliative toxin A (*eta*) and exfoliative toxin B (*etb*) have been cloned and sequenced (Lee *et al.,* 1987), showing 45% similarity at the amino acid level and have highly similar hydrophobicity/hydrophilicity profiles. Despite this similarity, the two proteins are antigenically distinct, with little cross-reactivity.

Exfoliative toxin produced by *S. hyicus* bound to GM4-like glycolipid extracted from the skin of 1-day-old chickens but did not bind to glycolipid from adult chickens or suckling mice. Exfoliative toxin produced by *S. aureus* bound to the GM4-like glycolipid extracted from the skin of suckling mice but not to glycolipid from 1-day-old or adult chickens. *S. hyicus* and *S. aureus* exfoliative toxins lost their toxicity by preincubation with GM4-like glycolipid from 1-day-old chickens and suckling mice, respectively (Tanabe *et al.,* 1995).

1.2.2.4 Alpha-haemolysin (alpha-toxin)

Alpha-haemolysin is by far the most intensively studied of the *S. aureus* cytotoxins. A high percentage of strains is reported to make this toxin, and it is toxic to a wide range of mammalian cells (Dinges *et al.,* 2000). It is particularly active against rabbit erythrocytes, and it is also dermonecrotic and neurotoxic. Levels of alpha-toxin as low as 1 µl are lethal when injected into rabbits intravenously. Extensive reviews of alpha-toxin have been published (Freer and Arbuthnott, 1982; Bhakdi and Tranum-Jensen, 1991).

The gene that encodes alpha-haemolysin, *hla*, was cloned from the *S. aureus* chromosome and sequenced (Gray and Kehoe, 1984). The first 26 residues of the translated polypeptide comprise a signal sequence, which is cleaved during secretion. The mature protein contains 293 residues and has a molecular weight of 33 kDa and a pI of about 8.5. There are no cysteines in the mature protein, which is composed primarily of β-sheets (65%), with α-helical structures making up a much smaller percentage (10%) of the secondary structure. This toxin is under control of the accessory gene regulator (*agr*) and is therefore made during the late exponential phase of growth in a batch culture (see section 1.4). It was observed by O'Reilly *et al.* (1990) that some strains contain the *hla* gene but do not produce detectable alpha-toxin.

Alpha-haemolysin was expressed phenotypically in 37% of the bovine isolates of *S. aureus*, in 59% of the human carrier isolates, and in 67% of the isolates from human septicaemia (Aaresstrup *et al.,* 1999). Gibbs *et al.* (1978b) have reported that some *S. aureus* poultry strains of phage types A (100%), B1 (36%), B2 (100%) and C (100%) were alpha-haemolysin producers.

1.2.2.5 Beta-haemolysin (beta-toxin, sphingomyelinase C)

S. aureus beta-haemolysin, first identified in 1935 by Glenny and Stevens, was also shown by them to be highly haemolytic for sheep but not rabbit erythrocytes. It is not dermonecrotic in guinea pigs, and it is not lethal in mice.

Glenny and Stevens (1935) also noted that the haemolytic activity of this toxin was enhanced by incubation below 10 °C after treatment at 37 °C, leading to the appellation 'hot-cold' haemolysin.

Beta-haemolysin is secreted into the culture medium as an exotoxin and has a molecular weight of 35 kDa. The gene was cloned by Coleman *et al.* (1986) and Projan *et al.* (1989), and the nucleotide sequence was published by Projan *et al.* (1989). The gene named *hlb*, is chromosomally-located on a 4-kb *ClaI* DNA fragment and encodes a 330-amino-acid polypeptide with a predicted molecular weight of 39 kDa. A 200-residue region of homology has been found to *Bacillus cereus* sphingomyelinase (55.7% similarity).

According to some reports, β-toxin is produced by 75-100% of *S. aureus* bovine strains and by 10-15% of human strains (Elek and Levy, 1950; Poutrel and Ducelliz, 1979). Aarestrup *et al.* (1999) reported that β-toxin was expressed by *S. aureus* in 72% of bovine, 11% of human carrier isolates and 13% of human septicemia isolates. Gibbs *et al.* (1978b) have reported that some *S. aureus* poultry phage type B1 (93%) and B2 (Variable) strains were β-toxin producers.

1.2.2.6 Gamma-haemolysin and PV-leukocidin (two-component toxins)

Two types of bicomponent toxins are made by *S. aureus*, gamma-haemolysin (γ-toxin) and Panton-Valenine (PV) leukocidin. Each of these toxins is made as two non-associated secreted proteins, referred to as S and F components (for slow- and fast-eluting proteins in an ion-exchange column) (Woodin, 1959; 1960). Gamma-haemolysin is reported to be made by virtually every human strain of *S. aureus*, while PV leukocidin is made by 2 to 3% of strains (Prevost *et al.,* 1995). The toxins affect neutrophils and macrophages, and gamma-haemolysin is additionally able to lyse many varieties of mammalian erythrocytes. Gamma-haemolysin is not identifiable on blood agar plates, however, due to the inhibitory effect of agar on toxin activity (Prevost *et al.,* 1995).

The genes for gamma-haemolysin are transcribed from a single locus, located on a 4.5-kb *ScaI*-digested chromosomal fragment. Extracts from a clone containing this fragment were haemolytic and leukotoxic. The open reading frames, in order, are named *hlgA, hlgC,* and *hlgB,* and they correspond to previously identified gamma-hemolysin gene (Couch *et al.,* 1988; Kamio *et al.,* 1993; Colin *et al.,* 1994). *hlgC* and *hlgB* are transcribed on a single mRNA, while *hlgB* is separately expressed. There is no information about gamma-haemolysin production in avian isolates of *S. aureus* isolates.

1.2.2.7 Delta-haemolysin (delta-lysin, delta-toxin)

Delta-haemolysin, a 26-amino-acid peptide, is capable of causing membrane damage in a variety of mammalian cells. Toxin from human isolates of *S. aureus* contain nine amino acid differences from the canine strain-derived delta-haemolysin although both are 26 residues long and have molecular weights of approximately 3 kDa. These different haemolysins have conserved charge properties, and both are haemolytically active, but only partial serological identity is observed. This protein is made by 97% of human *S. aureus* strains, and an immunologically related protein is made by *S. haemolyticus* strains. It is also hypothesized that 50-70% of coagulase-negative staphylococci produce this toxin (Bohach *et al.,* 1997). Although delta-toxin causes a wide range of cytotoxic effects, its importance in disease aetiology remains unclear. Delta-haemolysin is itself controlled by the accessory gene regulator (*agr*), and the highest expression in broth culture is found in the late-logarithmic (post-exponential) phase (Arvidson *et al.,* 1990).

Delta-toxin is capable of lysing erythocytes and other mammalian cells, as well as subcellular structures such as membrane-bound organelles, and also spheroplasts, and protoplasts (Freer and Birkbeck, 1982). Also, a dermonecrotic activity has been observed, as well as lethality in experimental animals when used in high concentrations. It remains possible, however, that these activities result from contamination with very small amounts of alpha-toxin. The presence

of phospholipids inhibits delta-toxin activity. Gibbs *et al.* (1978b) have reported that some *S. aureus* poultry strains of phage types A (100%), B1 (93%), B2 (60%) (40% of B2 isolates had variable results) and C (100%) were delta-toxin producers.

1.2.2.8 Lipase, fatty acid-metabolizing enzyme (FAME)

In response to an infection, the host can produce a variety of fatty acids and other lipid molecules that act as surfactants to disrupt the bacterial membrane, especially when an abscess is formed. Virtually all strains of staphylococci of human origin are lipolytic (Elek, 1959). Genes encoding lipases have been cloned from *S. aureus* (Lee and Landolo, 1986) and the non-pathogenic *S. hyicus* (Götz *et al.,* 1985), and it has been proposed that lipases have a negative effect on immune function. However, the definitive genetic inactivation experiments remain to be done. Lipases, like proteases, may well represent scavenging enzymes, one of whose roles is to harvest nutrients from the environment. An enzyme known as fatty acid-metabolizing enzyme (*FAME*) has been described (Götz *et al.,* 1985), that has been proposed to function to detoxify bactericidal fatty acids at the site of an infection. Consistent with a role in abscess formation, both lipase and *FAME* have been reported to be expressed extracellularly during the post-exponential phase. There is no information about the above genes in avian isolates of *S. aureus*.

1.2.2.9 Staphylocoagulase (coagulase)

Coagulase is a protein that clots plasma in the absence of Ca^{2+} but requires a plasma constituent known as coagulase-reacting factor (CRF). CRF, perhaps a derivative of prothrombin, reacts with coagulase, and the resulting coagulase-CFR complex (staphylothrombin) converts fibrinogen to fibrin (Drummond and Tager, 1963). A very high percentage (98-99%) of *S. aureus* strains exhibit coagulase activity (Kloos, 1998). Coagulase activity is also exhibited by the species *S. intermedius, S. hyicus, S. delphini* and *S. schleiferi* subsp. *coagulase.* The role of coagulase in virulence remains unclear. However, given the fact that

a large majority of strains of *S. aureus* produced this protein and that it binds to and activates prothrombin and causes coagulation of serum, it is likely to play a role in staphylococcal disease (Projan *et al.,* 1997). All poultry *S. aureus* strains examined, with one exception, rapidly coagulate rabbit and human plasmas, the remaining strain taking 24 h to coagulate human plasma. None coagulated bovine plasma as rapidly as the bovine control strains though a collapsed sac or flaky precipitate was often present after 24 h incubation. The lack of ability to coagulase bovine plasma appears to be a characteristic of poultry isolates (Harry 1967a, b; Hájek and Maršálek 1971; Bajljosov and Georgiev 1974), although Harry (1967a; b) also reported a flaky precipitate after incubating his avian isolates with bovine plasma for 18 h. On the other hand, Rodgers *et al.* (1999) reported that all *S. aureus* strains of the poultry biotype were negative for the ability to coagulate bovine plasma.

1.2.2.10 Staphylokinase

Staphylokinase, a protein that exhibits fibrinolytic activity indirectly by binding to plasminogen, is produced by a relatively high percentage (60-98%) of human *S. aureus* strains of biotype A, but by only a small percentage (5-10%) of bovine *S. aureus* strains of biotype C (Hájek and Maršálek, 1969). No substrate other than plasminogen has been found for staphylokinase. Activation of plasminogen to plasmin is specific and proceeds primarily through the cleavage of a single arginine-valine bond. Plasmin formed through activation of plasminogen with staphylokinase is indistinguishable from the plasmin formed with other activators streptokinase, urokinase or tissue-activators (Arvidson, 1983). Rodgers *et al.* (1999) reported that no *S. aureus* strains of the poultry biotype according to the schemes of Devriese (1984) and Isigidi *et al.* (1990), produced staphylokinase.

1.2.2.11 Bacteriolytic enzymes

The bacteriolytic enzymes endo-β-N-acetylglucosaminidase and N-acetylmuramyl-l-alanine amidase (Wadström and Hisatsune, 1970) are produced by *S. aureus* and many other staphylococcal species. The former enzyme is especially active against the cells of *Bacillus* and *Micrococcus* spp., which compete for some of the same cutaneous habitats as staphylococci. The bacteriolytic enzyme known as lysostaphin (or lysostaphin endopeptidase) is a zinc metalloenzyme that has as its main target the polyglycyl bridge of the cell wall peptidoglycan of staphylococci (Schindler and Schuhardt 1964; Trayer and Buckley, 1970) and salinicocci (Ventosa *et al.,* 1990). Lysostaphin is produced by *S. simulans* biovar staphylolyticus (Sloan, *et al.,* 1982). The plasmid containing the endopeptidase gene *end* also contains the lysostaphin endopeptidase resistance gene *epr* that specifies modification of the interpeptide bridge of the peptidoglycan, making the host *S. simulans* strain resistant to the lysostaphin endopeptidase that it produces (DeHart *et al.,* 1995). The modification of the interpeptide bridge involves a replacement of many of the glycine residues by serine residues. Most strains of *S. sciuri* produce a staphylolytic enzyme that is immunologically related to lysostaphin, but it is determined by a gene sharing little nucleotide sequence homology with *end* (Kloos *et al.,* 1997b). There is no information about bacteriolytic enzymes in avian isolates of *S. aureus.*

1.2.2.12 Staphylococcal nuclease (thermonuclease)

Both heat-stable and heat-labile nucleases are produced by staphylococci. A large percentage (95-100%) of the strains of *S. aureus, S. hyicus, S. intermedius* and *S. schleiferi* produce detectable levels of the heat-stable nuclease (or thermonuclease) (Devriese *et al.,* 1978; Freney *et al.,* 1988). Staphylococcal nuclease hydrolyses both DNA and RNA yielding predominantly 3′ and dinucleotide products. The enzymatic attack is both exonucleolytic and endonucleolytic (Arvidson, 1983). Gibbs and Patterson (1978 a, b) have reported

that all poultry *S. aureus* strains of phage types B and C were nuclease producers.

1.2.2.13 Urease

Urease activity is exhibited by many staphylococcal species, and it apparently plays a role in the invasiveness of *S. saprophyticus* in the urinary tract (Gatermann *et al.,* 1989). Mead *et al.* (1989) reported urease activity in avian *S. aureus* isolates in a comparison of strains from freshly slaughtered poultry and strains "endemic" to processing plants.

1.2.2.14 Hyaluronate lyase (hyaluronidase)

Hyaluronate lyase (hyaluronidase) is a glycoprotein produced by *S. aureus* and *S. hyicus* that hydrolyses the mucopolysaccharide hyaluronate (Rautela and Abramson, 1973). Pathogenic staphylococci that produce this enzyme have the capacity to break down hyaluronate-rich tissue barriers, thereby increasing their invasiveness. The *S. aureus* 8325-4 hyaluronate lyase gene (*hysA*) was identified after detecting hyaluronate lyase activity expressed by phages from a genomic library. The *hysA* open reading frame, capable of encoding a protein of 91980 Da, was identified by Tn5 mutagenesis and nucleotide sequencing. HysA shares 35 and 36% amino acid sequence identity with group B streptococcal hyaluronate lyase and pneumococcal hyaluronidase, respectively (Farrell *et al.,* 1995). There is no information about hyaluronidase in avian isolates of *S. aureus*.

1.2.2.15 Proteases

Most strains of *S. aureus* are proteolytic. This property is generally demonstrated by the use of gelatin or casein as substrates (Baired-Parker, 1965). Four major extracellular proteases are produced by strains of *S. aureus* (Arvidson 2000): staphylococcal serine protease (V8 protease) (SspA), a metalloprotease named aureolysin (Aur), a cysteine protease (Scp) named staphopain (Hofmann *et al.,* 1993), and a second cysteine protease (SspB) encoded within the same operon as

SspA (McGavin *et al.,* 1997; Chan and Foster, 1998). All four proteases appear to be synthesized as preproenzymes, which are proteolytically cleaved to generate the mature enzymes. In the case of the serine protease, the proform is enzymatically inactive and needs to be cleaved by aureolysin to become active (Drapeau, 1978). The proform of SspB that appeared to possess enzyme activity seems to be processed by SspA (Rice *et al.,* 2001). Which enzymes are involved in the processing of aureolysin and staphopain remains to be determined. The synthesis of extracellular proteases is positively regulated by *agr* and negatively regulated by *sarA* (Janzon *et al.,* 1986; Chan and Foster, 1998) (see section 1.4). Karlsson *et al.* (2001) have found that in *sarA* mutant cells, which produce high amounts of the four major extracellular proteases, the level of cell-bound fibronectin-binding proteins (FnBPs) and protein A were very low compared to those of wild-type cells, in spite of unaltered or increased transcription of the corresponding genes.

The serine protease has a serine residue in the active site and is inhibited by diisopropyl fluorophosphate. There is a single report of a protease produced by an avian strain of *S. aureus*, from a chicken suffering from dermatitis. Its activity was inhibited by thiol protease inhibitors, but was not inhibited by metalloprotease or serine protease inhibitors (Takeuchi *et al.,* 1999). The thiol protease produced by *S. aureus* strain V8 is active only in the presence of reducing agents and is inactivated by heavy metals. The metalloprotease produced by *S. aureus* strain V8 is completely inactivated by metal chelators, such as EDTA and 1, 10-phenanthroline (Drapeau *et al.,* 1972; Arvidson, 1973; Arvidson *et al.,* 1973). A second serine protease was noted by Kuroda *et al.* (2001) in the genome of two MRSA strains, which may have a function similar to serine protease (V8 protease) (SspA).

Genes encoding metalloproteases have been cloned from *S. hyicus* (Ayora and Götz, 1994; Takeuchi *et al.,* 2000) and *S. epidermidis* (Teufel and Götz, 1993), with the metalloprotease from *S. epidermidis* exhibiting elastase activity. The

serine protease known as V8 protease (from *S. aureus* strain V8) has the ability to cleave and inactivate antibodies and has been proposed to function in blocking the action of antibodies. A second role for proteases may involve protection against antimicrobial peptides such as the neutrophil defensins (Selsted *et al.,* 1993) or the platelet microbicidal proteins (PMPs) (Yeaman *et al.,* 1994). It has also been proposed that these proteases may not only function to inactivate key proteins and peptides involved in host defence but may also contribute to the destruction of tissue proteins and enhance invasiveness (Goguen *et al.,* 1995). Another logical role for these proteases is that they serve as molecular scavengers to obtain usable nutrients from the environment. The fact that many non-pathogenic bacteria found in the environment also elaborate similar proteases (e.g., *Bacillus subtilis*) suggests that this may be the case.

1.3 THE CHROMOSOME

Before the recent whole genome sequencing of a number of *S. aureus* strains (see section 1.3.4), one strain, NCTC 8325 (PS47), was most extensively studied. The NCTC 8325 strain belongs to phage group III and was obtained as a clinical isolate more than 40 years ago. The chromosome of this strain was mapped mainly by Pattee and coworkers (Pattee, 1990; 1993a; 1993b) by using genetic and physical methods. Genetic methods involved mutagenesis (formerly by chemicals and at present by transposons), transduction, transformation and cloning. Physical methods are based on the digestion of a genome with restriction enzymes that have a low number of recognized sites, e.g. *SmaI* and estimation of fragment length. These studies by physical mapping showed a great deal of similarity among the genomes of different *S. aureus* strain and the sizes were estimated as: NCTC 8325, 2.8 mbp; PS55 strain, 2.771 mbp; strain 112 (phage group I) 2.768 mbp; strain 42D (phage group IV) 2.652 mbp (Bannantine and Pattee, 1996; Iandolo *et al.,* 1997).

1.3.1 Mobile elements of the genome

Mobile elements of the bacterial genome include insertion sequences (*ISs*), transposons (*Tns*), gene cassettes and integrons. The first two possess genes responsible for their own transposition and most of them were classified by Sakaguchi (1990) as genetic elements named invertrons on the basis of the presence of short inverted repeats (*IRs*) at the ends. Gene cassettes and integrons were described originally in Gram-negative bacteria and, although strongly supposed to exist, they had not been found in staphylococci. Very recently however, some of these were found by Kuroda *et al.* (2001) in *S. aureus* strains N315 and Mu50 (see section 1.3.4). For mobilization and integration of gene cassettes, specific genetic systems, i.e. integrons, are needed (Recchia and Hall 1995). Cassettes can exist either free in a circular form or integrated with an integron at the *attI* site. An integron encodes a site-specific recombinase (integrase) that is responsible for the insertion of cassettes at *attI*, and also provides the promoter for expression of the cassette-encoded antibiotic resistance genes. Integrons have been found in several independent locations in different plasmids and transposons (Brown *et al.*, 1996).

A variety of mobile genetic elements has been described to be present in *S. aureus*: insertion sequence (*ISs*), transposons (*Tns*) and transposon-like elements. *ISs* are transposable elements that do not carry any genetic information except that required for transposition. They are present in many sites on the chromosome and on plasmids belonging to classes II or III. Although *ISs* do not code for any resistance, they are responsible for a recombination and stabilization of some resistance genes and their presence is very important in the evolution of the bacterial genome. Staphylococcal transposons possess one or two resistance markers beside genes responsible for transposition. Some of these transposons, e.g. *Tn551* or *Tn552*, are closely-related to the *Tn3* family of the II class of transposons. They have inverted terminal repeats (*ITR*) and transpose to more or less random sites creating duplicates of 5 or 6 nucleotides in target DNA. An interesting situation was observed in the case of the plasmid pIP1066

from *S. aureus*, where two closely located transposons *Tn552* and *Tn5404* possessed a common invertible fragment (3.5 kb). Orientation of this fragment alternatively determined if the *Tn552* on the right end, or *Tn5404* on the left is present (Derbise *et al.,* 1995). Within *Tn5404*, a third smaller transposon, *Tn5405* (related element to *Tn3854*) (Udo and Grubb, 1991) is located. *Tn5405* is flanked by *IS1182* (Derbise *et al.,* 1996). Other transposons like *Tn4001* are representatives of class I of transposons and are organized similarly to *Tn10.* Transposons of class I are flanked by identical *ISs* on both ends. One of the *S. aureus* transposons, *Tn554*, lacked the terminal inverted repeats and target duplications characteristic of other transposons.

1.3.2 Plasmids

Plasmids are common in natural populations of most staphylococcal species (Lacey, 1975; Kloos *et al.,* 1981; Weinstein *et al.,* 1982). They are rare (<2% of strains) in the species *S. auricularis* (Kloos, 1990) and relatively uncommon (<20% of strains) in *S. schleiferi* (Etienne *et al.,* 1990a) and members of the *S. sciuri* species group (Kloos *et al.,* 1981). The characteristics of many of the plasmids from *S. aureus* have been reviewed by Lyon and Skurray (1987) and Novick (1989). *S. aureus* plasmids have been classified into three classes, I, II and III. Class I plasmids are of small size (1-5 kbp) and have a high copy number (10-55 copies per cell), and usually encode a single antibiotic resistance or are cryptic. They have been subdivided into four subgroups or families on the basis of their nucleotide sequence and functional organisation (Rubby and Novick, 1975; Iordanescu and Surdeanu, 1980; Novick, 1989). Class II plasmids have been subdivided into two major families on the basis of incompatibility. These plasmids, commonly referred to as penicillinase or β-lactamase plasmids, are relativly large (15-33 kbp), have a low copy number (4-6 per cell), they carry various combinations of antibiotic and heavy metal resistance genes, many of which are located on transposons (Novick and Richmond, 1965; Sheehy and Novick, 1975; Shalita *et al.,* 1980). Class II plasmids, unlike class I plasmids, are very uncommon in the species *S. hyicus*, S. *intermedius,* S. *simulans* and S.

lugdunensis (Kloos *et al.,* 1981; Götz *et al.,* 1983; Etienne *et al.,* 1990a; 1990b). Most of the conjugative plasmids are class III plasmids that encode resistance to gentamicin and certain other aminoglycosides, such as tobramycin and kanamycin. These plasmids are relatively large (30-60 kbp). Class III plasmids have been identified also in *S. epidermidis* (Thomas and Archer, 1989).

Dodd *et al.* (1987; 1988) showed that plasmids were detected in 72% of strains of *S. aureus* isolates from different poultry processing plants but the proportion of plasmidless strains isolated varied amongst plants. Also plasmids were more common in strains of *S. aureus* characteristically associated with live poultry than with strains endemic in poultry plants and strains of human origin (Thompson and Holding, 1986).

1.3.3 Bacteriophages

Viruses infecting bacteria belong to 13 families (Murphy *et al.,* 1995). Known staphylococcal bacteriophages are members of two families of tailed phages: Myoviridae and Siphoviridae. Most naturally occurring *S. aureus* strains are multiply lysogenic. The temperate phages are usually inducible by UV and mitomicin C and typically integrate at unique chromosomal sites by the Campbell mechanism (Novick, 1990). All known temperate phages of *S. aureus* have a morphology typical of Siphoviridae (head and noncontractile tail). The bacteriophages of *S. aureus* were divided into 7 serological groups designated as A, B, C, D, F, G, L. Temperate bacteriophages, being most important for the variability of the bacterial genome, fall into three main serological groups, A, B and F. The majority of genetic investigations were performed using bacteriophage Φ11 (prophage of NCTC 8325 strain). Physical typing and genetic maps of this phage have been constructed (Krestschmer and Egan, 1975; Bächi, 1980).

The presence of a phage genome (prophage) can influence the bacterial cell and mediate lysogenic conversion (Smeltzer *et al.,* 1994). The first type of conversion leads to the inactivation of the bacterial gene caused by an

integration of the prophage in the gene (negative conversion). The best known examples of negative conversion in *S. aureus* take place when the integration site (*attB*) is localized within gene *hlb* (β-haemolysin) or gene *geh* (lipase). The *attB* sites of several prophages were mapped in different localizations of the NCTC 8325 strain chromosome: ΦM and Φ12 (*SmaI-A* fragment), Φ53 and Φ85 (*SmaI-B* fragment); ΦL54a, Φ77, ΦJ84 *SmaI-E*); Φ11, Φ13 and Φ42 (*SmaI-F*) (Berger-Bächi, *et al.,* 1989). The second type of conversion is positive and is mediated by an expression of prophage genes within the host cell. Some phages carry the genes of enterotoxin A (*sea*) or staphylokinase (*sak*) production or both (Mlynarczyk *et al.,* 1993; 1995a; 1995b). Additionally, expression of the structural genes of tails of some prophages can mediate competence for transformation and transfection (Lyon and Skurray, 1987). Moreover, some lysogenic bacteria acquire the ability of intracellular exchange of genetic material in mixed cultures (bacteriophage-mediated conjugation, a phenomenon quite different from transduction) (Lacey, 1972; Witte, 1981). Additionally, it has been shown that the presence of phage confers the loss of the chloramphenicol-resistance plasmid in *S. aureus* strains (Mlynarczyk *et al.,* 1981). Lysogenic conversion changes the susceptibility of *S. aureus* to phagocytosis (Mlynarczyk *et al.,* 1991) and is thought to cause tolerance to vancomycin (Mlynarczyk *et al.,* 1997).

1.3.4 Whole genome sequencing of *S. aureus*

The whole genome sequencing of two strains N315 and Mu50 of *S. aureus* (methicillin-resistant *S. aureus* and vancomycin-resistant *S. aureus*, MRSA and VRSA, respectively) has been reported recently by Kuroda *et al.* (2001). According to their websites the Institute for Genomic Research (TIGR) (http://www.tigr.org/tdb/mdb/mdbinprogress.html) is sequencing *S. aureus* strain COL and the University of Oklahoma (http://www.genome.ou.edu/staph.html) has completed the sequence (unpublished data) of *S. aureus* strain 8325. Another *S. aureus* strain EMRSA-16 is being sequenced by the Sanger Centre

(http://www.sanger.ac.uk/Projects/S_aureus) and *S. epidermidis* strains RP62A and ATCC12228 are being sequenced of TIGR and by the Chinese National Human Genome Centre at Shanghai (http://www.chgc.sh.cn/), respectively.

General features of *S. aureus* N315 and Mu50 genomes. N315 is a MRSA strain isolated in 1982, and Mu50 is an MRSA strain with vancomycin resistance isolated in 1997. The length of the genome of strain N315 is 2,813,641 bp and for strain Mu50 is 2,878,084 bp. The G+C content of strain N315 was 32.8% for the whole genome, 33.6% for the protein coding regions, 49.5% for the RNA coding regions and 27.7% for the non-coding regions. Corresponding values for strain Mu50 were very similar and there was 96% nucleotide sequence identity between the two strains. Mu50-specific mobile genetic elements represented most of the differences between the strains. Approximately 2,600 open reading frames for protein coding were identified. Other features of interest that were identified include different insertion sequences, transposons, bacteriophage and pathogenicity islands. Almost all of the known *S. aureus* pathogenic factors were identified within the genomes, as well as 70 new candidates. The latter included new genes for a possible staphylocoagulase, serine protease, thermonuclease, various possible toxins, haemolysins and superantigens.

Plasmid pN315 found in strain N315 is 24,653 bp and plasmid pMu50 in strain Mu50 is 25,107 bp. The antibiotic resistance genes identified in the N315 and Mu50 genomes are located on plasmids or mobile genetic elements.

1.4 VIRULENCE GENE REGULATORS OF *S. aureus*

In *S. aureus*, the expression of many virulence factors is co-ordinately regulated. The genetics of this regulation is largely understood in terms of the function of *agr* (accessory gene regulator) and *sar* (staphylococcal accessory regulator) genes (Novick, 1995). Together, the components encoded by these loci form part of a complex pathway that leads to decreased transcription of selected cell-

surface virulence factor genes and increased transcription of regulated extracellular toxins and enzymes (see **Figure 1.1**) (Janzon and Arvidson, 1990; Kornblum *et al.,* 1990; Novick *et al.,* 1990; Cheung and Projan, 1994; Heinrichs *et al.,* 1996). Translational control of alpha-toxin by the *agr-sar* system has also been demonstrated (Novick *et al.,* 1993; Morfeldt *et al.,* 1995). In *agr* or *sar* mutant strains, regulated cell-surface proteins (e.g., coagulase, fibronectin-binding protein, and protein A) are produced throughout the exponential and post-exponential phases of growth. This is in contrast to wild-type strains that only produce these proteins during exponential growth. Furthermore, in *agr* and *sar* mutant strains, many extracellular toxins and enzymes that are normally present in post-exponential phase cultures (e.g., alpha-toxin, metalloprotease, cysteine protease and serine protease) are reduced to as low as 5% of their normal levels (Janzon *et al.,* 1986; Janzon and Arvidson, 1990; Novick *et al.,* 1993; Chan and Foster, 1998; Karlsson *et al.,* 2001).

The *agr* locus encodes a self-inducing, pheromone-sensing, signal transduction circuit (**Figure 1.1**). One of two divergent *agr* messages is transcribed from a promotor designated P2 (Novick *et al.,* 1995). This message, RNA II, encodes four proteins, AgrA, AgrB, AgrC and AgrD. Two of these *agr*-encoded proteins: AgrC and AgrA, function as sensor and regulator proteins, respectively. The activating signal of the *agr* system is a pheromone encoded within the prepeptide protein AgrD. AgrB is believed to be the enzyme responsible for the maturation and/or secretion of AgrD pheromone (Ji *et al.,* 1995; 1997). AgrD serves as a peptide pheromone and is specifically recognised by the AgrC membrane-bound receptor. Once the extracellular concentration of AgrD reaches a particular level, AgrC initiates a signal transduction pathway that is believed to include AgrA. AgrA is thought to activate the expression of the *agr* operon (RNA II) and the divergently expressed RNA III. AgrA mutants have dramatically reduced virulence in animal models of staphylococcal arthritis, osteomyelitis, endocarditis and endophthalmitis (Abdelnour *et al.,* 1993; Cheung *et al.,* 1994a; Booth *et al.,* 1995; Gillaspy *et al.,* 1995). Mutations in any of the

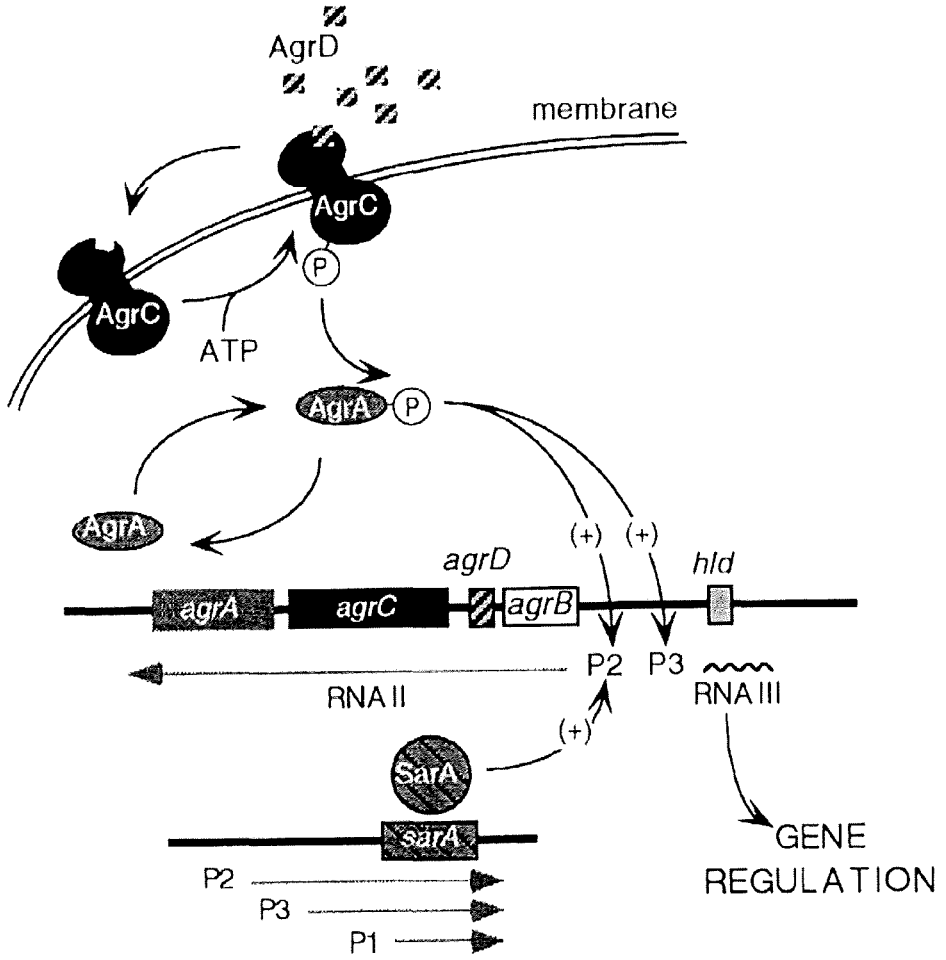


Figure 1.1 Model of the function of the agr-sar system. Chromosomal DNA is depicted as a thick black line. Promoters (P) are numbered and genes are shown as boxes. Phosphorylated proteins are associated with a circled letter P. The straight arrows and squiggly lines represent mRNA. The curved arrows show the relationships among the components of the system. Positive effect is marked with (+). *hld*: Delta-haemolysin gene (McNamara *et al.*, 2000).

agr open reading frames (ORFs A, B, C and D) eliminate the upregulation of RNA II and RNA III expression. The *agr* system up-regulates its function when AgrC binds the AgrD-derived signal (Novick, 1995). Like other bacterial sensor proteins, the binding of the signal results in the autophosphorylation of AgrC and, presumably, a concomitant activating conformational change (Lina *et al.,* 1998). The phosphate group on AgrC is then thought to be transferred to the regulator protein AgrA, resulting in the activation of AgrA.

Unlike other bacterial signal transduction systems in which the activated regulator protein directly initiates the transcription of target promoters, AgrA functions with the translation product of an unlinked locus named *sar* (Cheung *et al.,* 1995). The *sar* product, SarA, binds a region of DNA between the two *agr* promoters, and in conjunction with activated AgrA it up-regulates transcription of the *agr* messages (Cheung and Projan, 1994; Heinrichs *et al.,* 1996; Morfeldt *et al.,* 1996). The result of the increased transcription is an amplification of the circuit encoded by RNA II and high-level production of a 510-ribonucleotide message known as RNA III (Janzon and Arvidson, 1990; Novick *et al.,* 1993; Morfeldt *et al.,* 1996).

RNA III is the effector molecule of the *agr* response that upregulates transcription of many staphylococcal exoproteins and represses transcription of certain surface protein genes (Novick *et al.,* 1993; Novick *et al.,* 1995). In the case of alpha-toxin, a direct interaction between RNA III and the alpha-toxin message is required for full translation (Morfeldt *et al.,* 1995; 1996). When synthesised from a heterologous promoter in an *agr-null* mutant, RNA III returns a wild-type pattern of virulence factor messages and translation products (Vandenesch *et al.,* 1991; Morfeldt *et al.,* 1995).

Thus, the staphylococcal virulence gene system is activated by the density-sensing *agr* system, which is autoinduced by a short peptide (autoinducing peptide [AIP]) processed from a propeptide encoded by *agrD*. Four groups of

these AIPs, I, II, III and IV, have been described (Ji *et al.,* 1997, Jarraud *et al.,* 2000). A central segment of the N-terminal half of AgrC (the receptor), shows striking interstrain variation. This finding has led to the division of *S. aureus* isolates into three different *agr* specificity groups and to the division of non-*aureus* staphylococci into a number of other AIPs groups. The different AIPs cross-inhibit the *agr* responses between groups (Ji *et al.,* 1997, Jarraud *et al.,* 2000).

An additional layer of complexity in this model is added by the observation that SarA is transcribed on three different overlapping messages known from largest to smallest as A, C, and B (Bayer *et al.,* 1996). These messages are initiated from distinct upstream promoters named P1, P3, and P2, respectively (**Figure 1.1**). Each message ends at a common terminator downstream of the SarA open reading frame. The P1 and P2 promoters are dependent on σ^{SA}, the primary sigma factor in *S. aureus*, while the P3 promoter is dependent on σ^{SB}, a multiple-stress-responsive sigma factor (Vandenesch *et al.,* 1991; Kullik *et al.,* 1998). These data can be linked to the observation that virulence factor production is regulated by environmental conditions. For example, SarA message and levels of alpha-toxin are both elevated when the bacteria are exposed to oxidative stress (Cheung and Projan, 1994).

Despite the progress made in understanding the *agr-sar* system, the best available evidence suggests that additional regulatory factors are required for virulence factor production (Compagnone-Post *et al.,* 1991; Vandenesch *et al.,* 1991; Dassy *et al.,* 1993; Balaban and Novick, 1995; Bayer *et al.,* 1996; Wesson *et al.,* 1998; Rechtin *et al.,* 1999; Bischoff *et al.,* 2001). Very recent study has identified more loci directly or indirectly (by modulating *agr* expression) that regulate the expression of virulence genes. They are *sarR* (Manna and Cheung, 2001), *sarH1* (Tegmark *et al.,* 2000), *rot* (McNamara *et al.,* 2000), *seaSR* (Giraudo *et al.,* 1999) and *srrAB* (Yarwood *et al.,* 2001). Sigma B also modulates this multifactorial regulatory network by regulating the transcription

of SarA and *sarH1*. Kuroda *et al.* (2001) identified all of these regulators and two novel *sarA* homologues, *sarH2* and *sarH3*, in the *S. aureus* genomes. There is no information about the presence or the role of these regulatory virulence factors in avian isolates of *S. aureus*.

1.5 DISEASES CAUSED BY *STAPHYLOCOCCUS*

1.5.1 Pathogenicity of *S. aureus*

Certain of the cell surface adhesins, exocellular material and extracellular proteins produced by *S. aureus* are believed to play important roles in the pathogenicity of this organism. The species is well supported with accessory DNA elements carrying a variety of virulence and resistance factors, which act together with other mechanisms to make this organism one of the most successful opportunistic pathogens in man and animals. Infections caused by this species are often acute and pyogenic and, if untreated, may spread to surrounding tissue or to internal sites involving other organs. Some of the more common infections of the skin include furuncles or boils, cellulitis and impetigo.

1.5.2 Staphylococcosis in man

S. aureus is a common aetiological agent of post-operative wound infections, bacteraemia, pneumonia, osteomyelitis, acute endocarditis, mastitis, toxic shock syndrome and abscesses of the muscle, urogenital tract, central nervous system and various intra-abdominal organs. Food poisoning is frequently attributed to staphylococcal enterotoxins. Comprehensive reviews on the nature of human and animal model infections have been reported by Elek (1959), Cohen (1972), Jeljaszewicz (1973; 1976; 1981; 1985), Easmon and Adlam (1983), Wadström *et al.* (1994).

1.5.3 Staphylococcosis in animals

Many of the infections produced in animals are rather similar to those produced in humans. Staphylococcal mastitis in either a clinical or subclinical form may have considerable economic consequences in the dairy industry. *S. aureus* subsp. *anaerobius* is the aetiological agent of an abscess disease (caseous lymphadenitis) in sheep. *S. intermedius* is a common opportunistic pathogen of dogs and may cause otitis media, pyoderma, abscesses in various organs, mastitis, reproductive tract infections and purulent wound infections (Devriese and Hájek, 1980; Phillips and Kloos, 1981; Raus and Love, 1983; Szynkiewicz *et al.,* 1985). This species can also produce wound infections in humans following dog bites. *S. hyicus* is the aetiological agent of infectious exudative epidermitis (greasy pig disease) (Devrise and Hájek, 1980; Phillips and Kloos, 1981) and can also cause septic polyarthritis in pigs (Phillips *et al.,* 1980), skin lesions in cattle and horses (Devriese and Derycke, 1979; Devriese *et al.,* 1983), osteomyelitis in cattle and poultry (Carnaghan, 1966; Wise, 1971; De Kesel and Devriese, 1982), and may on occasion be associated with mastitis in cattle (Devriese and De Keyser, 1980). *S. delphini* has been implicated in purulent skin lesions of dolphins, external otitis, cystitis and abscesses and wound infections in the cat (Igimi *et al.,* 1989). *S. schleiferi* subsp. *coagulans* can produce external otitis in dogs (Igimi *et al.,* 1990).

1.5.4 Pathogenesis of staphylococcal infections in birds

All avian species are susceptible to staphylococcal infections. Coagulase-positive isolates of *S. aureus* are considered to be pathogenic for poultry, while coagulase-negative strains are thought to be non-pathogenic.

1.5.4.1 Transmission

For infection to occur, a breakdown in the natural defence mechanism of the host must occur (Anderson, 1986). In most cases, this would involve damage to an environmental barrier, such as a skin wound or inflamed mucous membranes. *S. aureus* enters through the breached barrier and travels to internal locations

where a locus of infection (e.g., osteomyelitis) is established, usually in the metaphyseal area of a nearby joint (Nairn, 1973; Daum *et al.,* 1990). In newly hatched chicks, the open navel provides a portal of entry leading to omphalitis and other types of infections. Minor surgical procedures (e.g., trimming of toes, beak, or comb; removal of the snood) and parenteral vaccinations may offer additional means of entry for staphylococci.

Another type of host defence impairment occurs following infectious bursal disease (Santivatr *et al.,* 1981), chicken infectious anaemia, or possibly Marek's disease, a virus infection where the bursa of Fabricius or thymus is damaged and the immune system is compromised. Under these conditions, septicaemic staphylococcal infections can occur and cause death. Gangrenous dermatitis caused by *S. aureus*, along with *Clostridium septicum*, can be seen following early infectious bursal disease virus infection (Frazier *et al.,* 1964; Rosenberger *et al.,* 1975).

Escherichia coli was discovered to be the predominant bacterial organism in livers of turkeys immediately following challenge with virulent haemorrhagic enteritis virus (HEV). However, when livers of survivors were cultured 2 weeks post-exposure, *Staphylococcus* spp. were the predominant bacteria (Newberry *et al.,* 1994). This suggests that HEV, and possibly other similar viral intestinal infections may create portals of entry and provide the underlying basis for subsequent staphylococcal problems associated with older, commercial turkeys.

Susceptibility to staphylococcal infections also may be influenced genetically. Two related lines of New Hampshire chickens had significant differences in mortality following experimental infection (Cotter and Taylor, 1991).

1.5.4.2 Incubation period

The incubation period is short. In experimental chickens, clinical signs were evident 48-72 h following intravenous inoculation. Experimentally, chickens can

be readily infected by the intravenous route, but not by the intratracheal or aerosol routes. The number of bacteria affects the ability to produce experimental disease consistently; at least 10^5 organisms/kg body weight are necessary (Mutalib *et al.,* 1983a; 1983b).

1.5.4.3 Signs

Early clinical signs include ruffled feathers, limping on one leg, drooping of one or both wings, reluctance to walk, and fever (Mutalib *et al.,* 1983a). Severe depression and death can follow. Birds surviving the acute disease have swollen joints, sit on their hocks and keel bone, and are reluctant or unable to stand (Emslie *et al.,* 1983; Mutalib *et al.,* 1983a). Clinical signs of septicaemic staphylococcal infection and gangrenous dermatitis occur in birds in good condition, and may only be evident because of increased mortality in the flock (Frazier *et al.,* 1964; Bickford and Rosenwald, 1975; Rosenberger *et al.,* 1975).

1.5.4.4 Morbility and mortality

Morbidity and mortality due to staphylococcosis is usually low, unless there has been massive contamination of chicks because of exposure to unusually high numbers of bacteria in the hatchery through environmental contamination, vaccination, or servicing procedures. Leg disorders are one of the most prevalent problems observed in broilers and turkeys. With the advent of further processing and need for birds with more breast meat, leg problems have become more prevalent. Several reports from diagnostic laboratories have indicated that *S. aureus* is the most common bacterial agent isolated from affected legs and joints (Kibenge *et al.,* 1982b).

Morbidity and mortality from septicaemic staphylococal infection are usually low. The number of chickens that develop gangrenous dermatitis is low, but usually all that develop lesions succumb to the infection (Frazier *et al.,* 1964; Bitay *et al.,* 1984).

1.5.5 Diseases caused by avian *S. aureus*

Staphylococcosis in poultry and other avian species has been recognised for over 100 years; most early reports describe arthritis and synovitis. *S. aureus* infections are common in poultry; the most frequent sites being bones, tendon sheaths, and joints of the leg (**Table 1.2**). Staphylococcal infections occur less frequently in other locations including skin, sternal bursa, yolk sac, heart, vertebrae, eyelid, and as granulomas in the liver and lungs. Staphylococcal septicaemia, causing deaths in laying birds, seems to be prevalent in hot weather and resembles fowl cholera (Skeeles, 1997).

Subdermal abscesses: These conditions are seen most commonly affecting the feet (bumblefoot) and sternal bursa and occur most frequently in mature birds, particularly those of the heavy breeds. There is abscess formation with swelling, heat and usually some pain. In bumblefoot, the under-surface of the foot is first affected and the lesion may then spread to involve the whole foot. There is caseation and necrotic tissue and some haemorrhage. The sternal bursa may be similarly affected but less frequently and less severely (Jordan and Pattison, 1996).

Gangrenous dermatitis: This may be seen in birds of all ages but most commonly in broiler chickens. The wing tips and the dorsal pelvic region are the sites most frequently affected and the lesions are dark, moist and gangrenous in appearance with crepitation. Staphylococci are often associated with *Cl. perfringens* type A, which may be the primary pathogen. Immunosuppression resulting from damage to the bursa of Fabricius predisposes to the condition in growing chickens (Jordan and Pattison, 1996).

Arthritis and tenosynovitis: These conditions may be seen in birds only a few weeks old but more commonly occur at about 7-12 weeks in chickens and 9-12 weeks in turkeys. In broiler breeders, stress caused by uneven feed distribution or coccidiosis may well predispose to an increase in cases of staphylococcal

Table 1.2 Staphylococcal infections in poultry

Location	Age	Lesion	Usual outcome
Bone	Any, usually older	Osteomyelitis	Lameness
Joint	Any, usually older	Arthritis/Synovitis	Lameness
Yolk sac	Chick, poults	Omphalitis	Death
Blood (septicaemia)	Any	Generalized necrosis	Death
Skin	Young	Gangrenous dermatitis	Death
Feet	Mature	"Bumblefoot"	Lameness

From Skeeles (1997)

infection. The affected joints, usually the hocks (tibiotarso-tarsometatarsal), are hot swollen and painful and affected birds are usually depressed, lame and reluctant to walk. When the phalangeal joints are affected, subdermal staphylococcal abscesses ("bumblefoot") may occur. The synovial membranes of joints and tendon sheaths (commonly in the region of the hock and feet) become thickened and oedematous and exudate with fibrin deposits is produced within and around the joints and around the tendon sheaths. There is some necrosis and exudate may later become caseous. Petechiae and larger haemorrhages may be present in the early stages. If the bird survives, the condition becomes chronic with the formation of fibrous tissue (Jordan and Pattison, 1996).

Yolk sac infection: *S. aureus* was isolated from chicks below ten days of age showing yolk sac infection. The yolk sacs were found distended and contained foul smelling yolk material and curdled material and the colour also varied from greenish yellow to dark brown (Lakshmamna-Char *et al.,* 1987).

Staphylococcal septicaemia, endocarditis and granuloma: Septicaemia is perhaps most commonly a sequel to a primary local staphylococcal focus. It is relatively rare and may result in sudden death with marked congestion of the carcass or initial depression and the appearance of haemorrhages and necrotic foci in the liver, lungs, spleen and myocardium. In chronic cases these may develop into granulomas with loss of weight. Endocarditis may also be a sequel and vegetations, particularly affecting the left atrioventricular valves, are seen at necropsy. Following experimental intravenous infection with this organism emaciation may occur without any specific gross lesions (Jordan and Pattison, 1996).

Spondylitis and osteomyelitis: *S. aureus* can cause abscesses in the bodies of the fifth to seventh thoracic vertebrae with periostitis and osteomyelitis and consequent pressure on the spinal cord resulting in paralysis. The head of the

femur, tibiotarsus and occasionally other bones may also be affected with osteomyelitis (Jordan and Pattison, 1996).

1.5.6 Diseases caused by avian *S. hyicus*

S. hyicus has been associated with fibrinoheterophilic blepharitis in chickens and turkeys (Cheville *et al.,* 1988) and was isolated from five of nine tibiotarsal growth plates of turkeys with stifle joint osteoarthritis (Tate *et al.,* 1993).

1.5.7 Prevention and control

1.5.7.1 Management procedures

Any management procedure reducing damage to host defence mechanisms will help prevent staphylococcosis. Because wounds are a portal of entry for *S. aureus* into the body, anything reducing the chance of injury will help prevent infection. Sharp objects such as splinters, jagged rocks, or metal edges that cut or puncture feet should be eliminated from areas where poultry are reared. Maintenance of good litter quality will reduce foot pad ulceration. Particular attention should be given to hatchery management and sanitation. *S. aureus* is found everywhere, and conditions in incubators and hatchers are ideal for bacterial growth. Recently hatched and hatching chicks with open navels and an immature immune system can easily be infected, leading to mortality and chronic infections shortly after hatching. Prevention of early infections with infectious bursal disease virus and chicken infectious anaemia virus will also help prevent staphylococcosis (Santivatr *et al.,* 1981).

1.6 MOLECULAR APPROACHES TO MICROBIAL IDENTIFICATION AND TYPING

1.6.1 Introduction

It has been observed that random isolates of the same species often differ in

multiple characteristics, whereas the progeny of any particular isolate are typically indistinguishable or highly similar. The central hypothesis of typing studies is that a set of isolates obtained from an epidemiological cluster or during the course of an infection in a single patient are, in fact, directly descended from a common precursor and, as such, will share characteristics by which they can be differentiated from unrelated isolates.

1.6.1.1 Biotyping

Biotyping refers to the pattern of metabolic activities expressed by an isolate and may include specific biochemical reactions, colonial morphology, and environmental tolerances (e.g., the ability to grow on certain media or at extremes of pH or temperature). Such characteristics have classically been used for taxonomy. In many clinical microbiology laboratories, biotyping is now routinely and reliably performed using automated or modular systems designed for species identification. Attempts to use biochemical reactions for strain differentiation of *S. aureus* and of coagulase-negative staphylococci have proved largely unsatisfactory both because of poor reproducibility among independent isolates representing the same strain and relatively weak discriminatory power (Mickelsen *et al.,* 1985; Tenover *et al.,* 1994).

When strains of *S. aureus* isolates from poultry were biotyped according to the scheme of Hájek and Maršálek (1973), they were found in three groups: biotype B, similar to other strains of pig or poultry origin; biotype A, similar to strains of human origin; and intermediate strains which could not be assigned to either of the other biotypes. The biotyping scheme of Hájek and Maršálek (1973) is shown in **Table 1.3**. A more recent biotyping scheme, that has been applied to isolates from N. Ireland that were used in the present study, is shown in **Table 2.7**.

Table 1.3 The classification of *S. aureus* strains from poultry according to Hájek and Maršálek (1973)

Biotype	Fibrino-lysin [a]	Pigment [b]	Coagulation of plasma		Haemolysin		CV-agar test [c]
			human	bovine	α	β	
A	+	+	+	-	+	±	-
B	-	+	+	-	+	±	+
Inter-Mediate	±	+	+	±	+	±	±

Fibrinolysin [a]: This enzyme, also call staphylokinase. Pigment [b]: Colonies cream, cream-yellow or yellow, positive. CV-agar test [c]: Crystal violet agar test.

1.6.1.2 Phage typing

Bacteriophage typing proved to be a useful method in studying the ecology of *S. aureus* and the epidemiology of the disease due to this organism. The finding that the international sets of phages for typing human and bovine *S. aureus* strains had limited application to poultry strains of *S. aureus* led to the establishment of separate phage sets specific for *S. aureus* from poultry sources (Shimizu, 1976; 1977b; 1978; Gibbs *et al.,* 1978a). Gibbs *et al.* (1978b) in Northern Ireland developed a diverse phage typing set for poultry strains of *S. aureus* consisting of 22 phages (Gibbs' set). In Japan, Shimizu (1977a) developed an avian phage typing set consisting of 25 phages (Shimizu's set). The set has been successfully used on chicken strains isolated from Czechoslovakia, England, Bulgaria, Belgium, Italy, Argentina, Australia and Japan (Hájek and Horák, 1978; Terzolo and Shimizu, 1979; Shimizu, 1979; Kibenge *et al.,* 1982a). Thompson *et al.* (1980a) used another phage typing method in their laboratory. They showed that their basic poultry phage set was able to type 80% of *S. aureus* strains isolated from diseased and healthy chickens or from the poultry environment in several countries (Germany, France, England, Belgium, Bulgaria, Argentina and Japan). Also, the Thompson's set has been successfully used on chicken strains isolated from Germany (Hentschel and Kusch, 1980) and Bulgaria (Grigorova *et al.,* 1982).

Shimizu *et al.* (1986) used Shimizu and Gibbs' phage sets for typing 295 *S. aureus* strains isolated from diseased and healthy chickens, a turkey, pigeons and birds of prey in Japan and 5 other countries. Typability with Shimizu's set of 25 phages and with Gibbs' set of 22 phages was compared. Of 295 strains, 230 (78%) strains were typable with the former and 238 (80.7%) strains were typable with the latter, respectively. The majority of the poultry strains were highly susceptible at routine test dilution to the phages in the two sets. When the two typing sets were combined, the increase of typability was ca. 10%.

One obvious problem in applying a phage typing system on a national or international basis is its effectiveness in lysing strains from different geographic regions (Shimizu, 1978). Bacteriophage typing is not a static system, as it must respond to the emergence of new strains of staphylococci that are nontypable by the latest set of phages (Kloos and Lambe, 1991). In some studies, phage typing is only performed at 100 X RTD (routine test dilutions) and is reported to show "increased sensitivity" because "more distinct types are resolved". The significance of defining more "distinct types" among unrelated isolates can be difficult to interpret unless the reproducibility of the methodology has been fully assessed (Hunter, 1990). Simply generating more types does not mean the discriminatory power has improved. In fact, in some instances, the observation that phage typing systems produce illogical or inconsistent results may indicate problems in methodology or interpretation (Kristjánsson *et al.,* 1994). Studies using phage typing also document the extent to which ongoing variation may complicate attempts to track epidemic strains. With the emergence of MRSA as both a major epidemiological problem and a relatively common class of nontypable isolates, phage typing became increasingly unsatisfactory (Parker, 1983).

1.6.2 Molecular techniques

Macromolecules like nucleic acids, proteins, and lipopolysaccharides (in Gram-negative bacteria) carry important information about a particular organism. The sequence and organisation of these macromolecules should provide a universal molecular approach to microbial identification and typing of any organism. Recently developed techniques used to isolate and characterise macromolecules in molecular biology have become increasingly rapid and simple, to the extent that they are now readily available for any laboratory. Although the examination of protein and lipopolysaccharide profiles has been used to type a variety of different bacteria, there is a limitation to these techniques in that they analyse the phenotype rather than the genotype of a particular organism. The analysis of the genotype of an organism does not depend on phenotypic variation. Thus,

genotypic characterisation of bacteria is superior to phenotypic characterisation (Kerr, 1994). Some of the molecular techniques that have been used for identification and typing of *S. aureus* strains are outlined below.

1.6.2.1 Protein analysis

Proteins play a role of structural and metabolic function in any organism. The diversity of these proteins among organisms provides potential identification and typing systems. Several techniques have been applied to detect protein profiles. Electrophoretic protein typing is performed by isolating materials, including proteins and glycoprotein conjugates from whole cell or cell surface preparations, separating the materials by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and staining the proteins in the gel to determine the resulting pattern. These techniques have been used in epidemiological investigations of *S. aureus* (Gaston *et al.,* 1988; Mulligan *et al.,* 1988). For example in the study by Gaston *et al.* (1988) three electrophoretic methods of typing methicillin-resistant *S. aureus* (MRSA) strains, namely plasmid profiles (PP), whole-cell protein profiles (WCPP) and immunoblotting profiles (IP), were evaluated and compared with phage typing. The results obtained with isolates from 12 outbreaks were compared both within the outbreaks, to determine the consistency of results, and between outbreaks. There was generally good agreement between the typing methods but in only six outbreaks did all four methods indicate the same relationship between isolates. It was concluded that both WCPP and IP could provide valuable epidemiological data on MRSA and that IP was the easiest of the three methods to interpret.

However, it is important to remember that the electrophoretic patterns of cell proteins are usually highly complex and the profiles generated may vary according to growth conditions and the method of sample preparation.

1.6.3 Nucleic acid analysis

With the advent of molecular biology, strain typing has become more focused on DNA-based methods. Initial techniques compared restriction endonuclease patterns of chromosomal or plasmid DNA. The next generation of genotyping methods has included Southern blot hybridisation using gene-specific probes, ribotyping, polymerase chain reaction (PCR)-based approaches, pulsed-field gel electrophoresis (PFGE) for typing of *S. aureus* isolates (Bannerman *et al.,* 1995; McCullagh *et al.,* 1998), and multilocus sequence typing (MLST) (Enright *et al.,* 2000). These methods require interpretation and comparison of patterns and fingerprint images. The ability to digitise and store images and to compare patterns by using matching software programmes has enhanced these methods.

1.6.3.1 Analysis of plasmid DNA

Bacterial plasmids are autonomously replicating extrachromosomal genetic elements that are normally quite distinct from the chromosomal genotype that defines the host strain. Plasmids can be acquired by a variety of mechanisms and, although often stably inherited as the host strain proliferates, they can be lost spontaneously, as they are not generally essential for the survival of the host cell (Archer *et al.,* 1985; John and Twitty, 1986; Woodford *et al.,* 1993).

Plasmid profile analysis is technically simple and represents the first DNA-based typing method applied to *S. aureus* (McGowan *et al.,* 1979; Locksley *et al.,* 1982). The number and size of the plasmids carried by an isolate are determined by preparing a plasmid extract and subjecting it to routine agarose gel electrophoresis (Mayer, 1988). The technical reproducibility of plasmid profiles is confounded by the fact that a plasmid can exist in different molecular forms, that is, supercoiled (closed circle), nicked (open circle), linear, and oligomeric, each of which migrates differently during agarose gel electrophoresis. Thus, in different preparations, there can be variation in both the number and relative intensity of bands representing each individual plasmid (Zuccarelli *et al.,* 1990). Both the discriminatory power and technical reproducibility of plasmid analyses

can be improved by digesting the plasmids with restriction enzymes and analysing the number and size of the resulting restriction fragments (Mayer, 1988).

For further information on plasmids in staphylococci, see Section 1.3.2.

1.6.4 Analysis of chromosomal DNA

Restriction endonuclase analysis (REA). In conventional restriction enzyme analysis (REA), bacterial DNA is digested with endonucleases that have relatively frequent restriction sites, generating hundreds of fragments ranging from approximately 0.5 to 50 kb in length. Different strains have different REA profiles because of variations in their DNA sequences that alter the number and distribution of restriction sites. All isolates are typable by REA; however, the profiles consist of hundreds of bands that may be unresolved and overlapping, and therefore difficult to compare e.g. for *Pseudomonas aeruginosa* (Ogle *et al.,* 1987) and for coagulase-negative staphylococci (Bialkowska-Hobizanska *et al.,* 1990). In addition, the patterns may be confounded by restriction fragments derived from plasmids, which can readily contaminate genomic DNA preparations. Thus, isolates that differ only in their plasmid content may be designated as different strains. The analysis of independent plasmid preparations coupled with computer-assisted subtraction techniques can compensate for this effect (Bialkowska-Hobizanska *et al.,* 1990); however, such laborious procedures are rarely implemented. In general, REA has been supplanted by other genotyping methods.

1.6.4.1 Hybridisation procedures

Nucleic acid hybridisation is done using a labelled nucleic acid probe. There are two steps involved in the hybridisation technique. First, the target nucleic acid molecule is denatured by treatment with alkali or heating and then a labelled nucleic acid probe is annealed by subsequent neutralisation or cooling. The specificity (stringency) and speed of such a hybridisation reaction can be

controlled by manipulating the temperature, salt concentration, pH, probe concentration and probe size. The greater the stringency, the greater the specificity. The probe can be a specific cloned DNA fragment or a synthetic oligonucleotide. The method of labelling of the probe depends on whether the detecting system is either direct or indirect. The direct labels are radioisotopes such as ^{32}P and ^{35}S, and indirect labels include enzymes such as alkaline phosphatase and horseradish peroxidase or dyes like ethidium, fluorescein or DIG labelling system. In indirect labelling, the probe is labelled with a modifying group which subsequently binds with a reporter molecule. The radioactive labels have restrictions to use i.e. they may have short half-lives and possible health and safety hazards. The hybridisation can be applied after purification of target DNA by agarose gel electrophoresis and transferring DNA to a solid (nitrocellulose or nylon membranes) matrix by Southern blotting or in liquid phase. In general, probe assays which are based on labels that provide signal amplification (i.e. enzymes) can be expected to be more sensitive than assays based on labels that provide only a single signal per label e.g. fluorescein. The main drawback is that each individual species to be identified requires its own specific probe and this is an identification method rather than a typing system. However, it can be developed into a typing method by combining with restriction endonuclease analysis (Towner and Cockayne, 1993).

Many different Southern blot analyses have been applied to typing *S. aureus* isolates (Tenover *et al.,* 1994). However, few systems, other than ribotyping, have been used widely, and none has been established as particularly effective.

1.6.5 PCR-based techniques

Nucleic acid amplification by PCR has been applied to the detection and identification of microbes and is becoming widely used for detection for diagnostic purposes in clinical research and diagnostic laboratories (Whelen and Persing, 1996; Vaneechoutte and VanEldere, 1997). PCR allows identification of low amounts of DNA and is highly specific since the amplification depends

on the use of primers with sequences which are complementary to the DNA molecule to be amplified. The PCR is not hindered by the presence of DNA from other sources and therefore allows the selective amplification of one DNA molecule with a predefined sequence in the presence of large amounts of other nucleic acids. The ability to specifically amplify DNA by PCR from low numbers of bacteria, as well as its simplicity, rapidity and reproducibility offers advantages over the conventional cultural and phenotypic methods for identification and differentiation of strains.

1.6.5.1 PCR-ribotyping

The 16S-23S rRNA intergenic spacer of the ribosomal RNA operon (*rrn*) has proved useful for identification of strains and species (Barry *et al.,* 1991; Jensen *et al.,* 1993; Gürtle and Stanisich, 1996). The spacer region is considered non-functional and is consequently argued to be under minimal selective pressure (Barry *et al.,* 1991). Its evolutionary rate is 10 times greater than that of 16S rDNA (Leblond-Bourget *et al.,* 1996), the sequence of which is routinely used for phylogenetic studies of bacteria (Ludwing and Schleifer, 1994). The high evolutionary rate makes it possible to distinguish closely-related bacterial species and strains.

PCR amplification of the intergenic spacer region, using primers complementary to conserved regions of the 16S and 23S genes, i.e. PCR ribotyping, generally produces multiple fragments for each isolate, with appreciable diversity in the pattern of fragments among a few epidemiologically-unrelated isolates (Dolzani *et al.,* 1994; Kostman *et al.,* 1995). However, there is considerable variation in the amount of each of the different fragments produced for a given isolate. Consequently, the patterns comprise mixtures of intense and faint staining bands, which may complicate comparisons of different isolates (Dolzani *et al.,* 1994). Appuhamy *et al.* (1998a, b) characterised the spacer region between 16S and 23S rRNA genes of two ribosomal operons, termed *rrn*A and *rrn*B of

Actinobacillus seminis isolates and used PCR-ribotyping to differentiate this organism from related species.

Jensen *et al.* (1993) have developed a unified set of primers and PCR conditions to amplify spacer regions between the 16S and 23S genes in the prokaryotes including *S. aureus* isolates. On the basis of the results obtained with their test group of 300 bacterial strains, amplification of the 16S-23S ribosomal spacer region is a suitable process for generating a data base for use in a PCR-based identification method, which can be comprehensively applied to the bacterial kingdom. A high level of intraspecies variation of 16S-23S spacer regions has been reported in *S. aureus* (Jensen *et al.,* 1993; Saruta *et al.,* 1997). Also, Forsman *et al.* (1997) determined the sequences of the 16S-23S spacer regions for bovine mastitis *S. aureus* and the eight *Staphylococcus* and *Streptococcus* species with specific primers pairs for these species and genera.

1.6.5.2 Randomly amplified polymorphic DNA (RAPD)-PCR

Welsh and McClelland (1990) and Williams *et al.* (1990) reported that organisms could be typed using PCR fingerprints with primers having random sequences. The application of this method in microbiology has been reviewed by Power (1996). It has been termed arbitrarily-primed PCR (AP-PCR) or random amplified polymorphic DNA assay (RAPD). For conventional PCR, knowledge of target sequence is a prerequisite and that drawback has been eliminated with RAPD-PCR. The basis of the method is that a single primer combined with two cycles of PCR at low stringency and many cycles at high stringency generate a discrete and reproducible set of amplification products characteristic of particular genomes. The theory for this method is that at a sufficiently low temperature a primer can anneal to many sequences with a variety of mismatches. Some of these sequences will be within a few hundred base pairs of each other and on opposite strands, such that the intervening sequences will be amplified by PCR (Welsh and McClelland, 1990). The primers used for RAPD

have varied in the number of nucleotides: 20 (Welsh and McClelland, 1990); 10-12 (Williams *et al.,* 1990): and as short as five (Caetano-Annolles *et al.,* 1991).

Meunier and Grimont (1993) have investigated the reproducibility of RAPD fingerprinting. They found that different brands of *Taq* DNA polymerase have drastic effects on the reproducibility of banding patterns of RAPD and that the cause of this variation was the buffer recommended by the manufacturer, not the *Taq* DNA polymerase. They also found that make of thermal cycler affected the reproducibility of the RAPD pattern. van Belkum and Meis (1994) reported that RAPD was a good PCR fingerprinting method that gave excellent resolution, a high degree of reproducibility, exquisite sensitivity and extreme versatility when compared to other typing techniques and that large numbers of strains can be analysed within a short period of time. Arbeit *et al.* (1994) however has pointed out that RAPD produces some bands by inefficient reactions, resulting in variation in amplification and generation of bands of different sizes and intensity that cause difficulties in comparing profiles. They reported that there are no standards for the reproducibility of RAPD and the discriminatory power of pulsed field gel electrophoresis is higher than RAPD. On the other hand, it has been reported that RAPD typing is far more sensitive than multilocus enzyme electrophoresis typing for discriminating between related strains of a species (Wang *et al.,* 1993). A comparative study of ribotyping, PFGE and RAPD with *Clostridium difficile* has shown that the discriminatory power and analysis of patterns is higher but time-consuming in PFGE than RAPD (Chachaty *et al.,* 1994).

van Belkum *et al.* (1995) have been leading contributors in evaluating AP-PCR for *S. aureus*. They concluded that AP-PCR is well suited for genetic analysis and monitoring of nosocomial spreading of staphylococci. However the interlaboratory reproducibility and standardisation of DNA-banding patterns remains a problem. Benito *et al.* (2000) used AP-PCR for rapid identification of *S. aureus* from other staphylococcal species.

1.6.5.3 Repeat element PCR

REP-PCR. Versalovic *et al.* (1991) described a method for fingerprinting bacterial genomes by examining strain-specific patterns obtained from PCR amplification of repetitive DNA elements present within bacterial genomes. The repetitive extragenic palindromic (REP) elements are 38-bp sequences consisting of six degenerate positions and a 5-bp variable loop between each side of a conserved palindromic stem (Stern *et al.,* 1984). DelVecchio *et al.* (1995) described a REP-PCR for the molecular genotyping of 170 MRSA *S. aureus* isolates. They concluded that REP-PCR of MRSA can easily determine an outbreak source, and it can have important clinical and epidemiological ramifications. It can be used in routine surveillance and can enhance the potential for infection control. This high-stringency PCR fingerprinting method is based on a repetitive sequence found in *Mycoplasma pneumoniae* (Wenzel and Hermann, 1988), but it also generates strain-specific DNA fragments when *S. aureus* DNA is used as an amplification template. Very recently Kuroda *et al.* (2001) has shown the presence of unique repetitive sequences in two human *S. aureus* genome sequences.

ERIC-PCR. The enterobacterial repetitive intergenic consensus (ERIC) sequences have also been targeted for bacterial typing. ERIC sequences are 126-bp elements which contain a highly conserved central inverted repeat and are located in intergenic regions of the bacterial genome (Sharples and Lloyd, 1990; Hulton *et al.,* 1991). They have been defined primarily on sequence data obtained from *E. coli* and *Salmonella typhimurium* (Sharples and Lloyd, 1990; Hulton *et al.,* 1991). Appuhamy *et al.* (1997) used both REP- and ERIC-PCR to distinguish bovine *Haemophilus somnus* isolates. In another study by Appuhamy *et al.* (1998a) REP- and ERIC-PCR were used for strain differentiation in an epidemiological study of *Actinobacillus seminis* isolates. van Belkum *et al.* (1993) reported that ERIC sequences have not been demonstrated to be present in *S. aureus* or other Gram-positive bacteria. However, Wieser and Busse (2000) used an ERIC-PCR method for rapid identification of *S. epidermidis* strains.

While the REP and ERIC sequences are the most commonly used targets for DNA typing, other repeat sequences have been used as the basis of DNA fingerprinting methods.

BOX repeat element PCR. The BOX sequence has been used to differentiate strains of *Streptococcus pneumoniae* (Martin *et al.,* 1992; Koeuth *et al.,* 1995; van Belkum *et al.,* 1996b). Box elements are located within intergenic regions and can also form stem-loop structures due to their dyad symmetry. They are mosaic repetitive sequences referred to as *boxA*, *boxB*, and *boxC* (Martin *et al.,* 1992). The three subunit sequences have molecular lengths of 59, 45 and 50 nucleotides long, respectively. There are no relationship between these elements and the REP and ERIC units (Martin *et al.,* 1992). While initially thought to be unique to *S. pneumoniae*, BOX elements have now been found in *Helicobacter pylori* (Kwon *et al.,* 1998) and Wieser and Busse (2000) used REP-, ERIC- and BOX-PCR for further genotypic characterisation of some human coagulase-negative staphylococci and *S. aureus*. ERIC- and BOX-PCR yielded a species-specific banding pattern for all *Staphylococcus epidermidis* strains. Furthermore, all staphylococcal reference strains investigated exhibited distinct banding patterns, clearly distinguishable from that of *S. epidermidis*. No species-specific banding patterns could be observed after REP-PCR. As species identification of coagulase-negative staphylococci by fatty acid analysis and biochemical tests is known to be difficult, ERIC- and BOX-PCR seem to be excellent tools for the identification of *S. epidermidis* strains.

1.6.5.4 Restriction fragment length polymorphism (RFLP) analysis of PCR products

PCR of specific chromosomal regions has been combined with restriction endonuclease digestion of the PCR products which are then resolved on agarose or polyacrylamide gels to produce **RFLP**s. This system has been successfully applied to differentiate strains of rickettsiae by amplifying the citrate synthetase gene (Regnery *et al.,* 1991). The advantages of this system are that small

quantities of chromosomal DNA are sufficient and the procedure takes only a short time. The coagulase gene of *S. aureus* shows a high level of allelic variation associated with a series of 81-bp tandem repeats, varying in number among different strains; the sequences of individual repeats are highly homologous but differ in the presence or absence of an *AluI* restriction site. *AluI* digests of this locus have proved highly reproducible but only moderately discriminatory for *S. aureus* strain typing (Goh *et al.,* 1992; Tenover *et al.,* 1994). The polymorphic X-region of the protein A gene (*spa*) consists of a variable number of 24-bp repeats and is located immediately upstream of the region encoding the C-terminal cell wall attachment sequence. PCR amplification of the X-region followed by restriction analysis was equally as reproducible and easy to implement in the laboratory as was RAPD and RNA spacer region amplification. It also allowed further subtyping of bovine mastitis isolates with identical PFGE patterns (Lange *et al.,* 1999). Frénay *et al.* (1996) demonstrated the discrimination of MRSA isolates by amplification of the X-region. Also, van Belkum *et al.* (1996a) showed that a Danish collection of *S. aureus* strains varied within the X-region of the protein A gene. They concluded that the X-region is hyper-variable and that the *spa* region has probably evolved independently from the remaining *S. aureus* genome.

1.6.6 Multilocus enzyme electrophoresis (MLEE)

In multilocus enzyme electrophoresis (MLEE), isolates are analysed for differences in the electrophoretic mobilities of a set of metabolic enzymes (Selander *et al.,* 1986). Cell extracts containing the soluble metabolic enzymes are electrophoresed in non-denaturing starch gels. For each enzyme analysed, a replicated gel, or slice of a gel, is stained with a specific colorimetric substrate, such that the position of the enzyme is detected by the appearance of a visible reaction product (Selander *et al.,* 1986). Such analysis of variation on the basis of differences at a limited number of proteins has been useful for estimating population structure and evolution (Enright *et al.,* 2000). MLEE is powerful tool for such population studies, because as each individual enzyme represents an

independent characteristic, the data are suitable for rigorous mathematical analysis (Selander *et al.,* 1986). However, MLEE method is generally considered tedious and time-consuming, since population genetic studies require the analysis of sizeable numbers of loci in large number of isolates (Combe *et al.,* 2000). Also, the techniques and equipment are somewhat specialized and are available in relatively few laboratories. Consequently, the method has had relatively limited application to epidemiological studies (Tenover *et al.,* 1994; Maslow *et al.,* 1995).

MLEE has proved to be only moderately discriminatory for the epidemiological analysis of clinical isolates of *S. aureus* and other species. Sixty-three *S. aureus* isolates recovered from bovine sources in the USA and the Republic of Ireland were characterised by MLEE, ribotyping and random amplified polymorphic DNA PCR (RAPD-PCR) typing at two separate laboratories. The *S. aureus* isolates were assigned by MLEE to 10 electrophoretic types (ETs) (index of discrimination, D = 0.779). In contrast, the same isolates were assigned to 13 ribotypes (D = 0.888) and to 12 RAPD types (D = 0.898). *S. aureus* clones recovered from cows in Ireland were also associated with mastitis in dairy cows in the USA. These finding were consistent with the hypothesis that only a few specialised clones of *S. aureus* are responsible for the majority of cases of bovine mastitis, and that these clones had a broad geographic distribution (Fitzgerald *et al.,* 1997).

1.6.7 Pulsed-field gel electrophoresis (PFGE)

This method, developed by Schwartz and Cantor (1984), is a variation of agarose gel electrophoresis in which the orientation of the electric field across the gel is changed periodically ("pulsed"), rather than being kept constant as in conventional agarose gel electrophoresis used for the REA and Southern blot studies. This critical modification enables DNA fragments as large as megabases to be separated effectively by size (Birren and Lai, 1993). Suitable unsheared DNA is obtained by embedding intact organisms in agarose plugs ("inserts"),

and enzymatically lysing the cell wall and digesting the cellular proteins. The isolated genomes are then digested in situ with restriction enzymes that have few recognition sites (Arbeit *et al.,* 1990; Maslow *et al.,* 1993).

When a staphylococcal chromosome of about 2,800 kb, with a G+C content of approximately 34% is digested with *SmaI* (recognition sequence CCCGGG), PFGE analysis provides a chromosomal restriction profile composed of 15 to 20 distinct, well-resolved fragments ranging from approximately 10 to 800 kb. PFGE was more efficient at differentiating strains of methicillin-resistant *S. aureus* than other methods tested such as random amplified polymorphic DNA (RAPD) (Saulnier *et al.,* 1993).

Many of the strains used in the present work were provided by Dr. McCullagh and had been typed by PFGE. McCullagh *et al.* (1998) used the PFGE method to investigate the epidemiology of *S. aureus* infection in N. Ireland commercial broiler flocks and to show that the majority of the strains originated from the hatcheries. The resultant banding patterns were visually compared to a control included on each gel. The control selected, strain 24, was obtained from a case of proximal femoral degeneration (PFD) in broilers. The bacterial grouping was based on the number of similar bands present between restriction fragment patterns. The groups consisted of strains (a) identical to strain 24 type, showing no band differences, (b) closely related, showing 1-3 band differences, (c) possibly related, showing 4-6 band differences and (d) unrelated, showing more than 6 band differences. The results of the clinical studies of *S. aureus* isolates from broiler farms showed that the majority of these strains (60%) were from leg infections i.e. bone, joint or hock, 34% were from liver and the remainder were from wattles (6%). PFGE typing for strains isolated from clinical disease of broiler chickens, compared with strain 24, showed that 57% were identical, a further 28% showed relatedness to strain 24 and only 15% were unrelated. Also the results of isolates from hatcheries showed 48% strains were identical, 22% showed relatedness and 29% were totally unrelated to strain 24.

PFGE has two notable limitations. First, because of the need to diffuse all buffers and enzymes into the agarose insert, the preparation of suitable DNA involves several extended incubations and takes 2 to 4 days (Maslow *et al.,* 1993). This can reduce the laboratory's ability to analyse large numbers of samples. Second, PFGE requires relatively expensive specialised equipment (Birren and Lai, 1993).

1.6.8 Multilocus sequence typing (MLST)

MLST is a recent and highly discriminatory method of characterising bacterial isolates, on the basis of the sequences of ~450 bp internal fragments of, usually, seven housekeeping genes (Maiden *et al.,* 1998). For each gene, the different sequences are assigned as alleles and the alleles at the seven loci provide an allelic profile, which unambiguously defines the sequence type (ST) of each isolate. MLST has been used for the identification of the hypervirulent lineages of *Neisseria meningitidis* (Maiden *et al.,* 1998) and for assigning *Streptococcus pneumoniae* strains to the major hypervirulent clones (Enright *et al.,* 1998; Enright *et al.,* 1999). MLST has also been applied to *S. aureus*. The sequences of internal fragments of seven housekeeping genes were obtained for 155 *S. aureus* isolates from patients with community-acquired and hospital-acquired invasive disease in the Oxford area, United Kingdom. Fifty-three different allelic profiles were identified, and 17 of these were represented by at least two isolates (Enright *et al.,* 2000).

1.6.9 DNA microarrays

It is now possible to index diversity in overall chromosomal gene content in isolates of a bacterial species. For example, whole-genome DNA microarray analysis has been used to compare the genomes of 13 variants of the tuberculosis vaccine strain, bacillus Calmette-Guérin, and *Mycobacterium tuberculosis* H37Rv (Behr *et al.,* 1999). Fitzgerald *et al.* (2001) reported a DNA microarray representing >90% of the *S. aureus* genome to characterise genomic diversity, evolutionary relationships, and virulence gene distribution among 36 human,

bovine and ovine isolates of divergent clonal lineages, including methicillin-resistant isolates and organisms causing toxic shock syndrome. Genetic variation in *S. aureus* is very extensive, with ~22% of the genome comprised of dispensable genetic material. Eighteen large regions of difference were identified, and 10 of these regions have genes that encode putative virulence factors or proteins mediating antibiotic resistance. Also they found that lateral gene transfer has played a fundamental role in the evolution of *S. aureus*. The *mec* gene has been horizontally transferred into distinct *S. aureus* chromosomal backgrounds at least five times, demonstrating that methicillin-resistant strains have evolved at multiple, independent times, rather than from a single ancestral strain. This finding resolves a long-standing controversy in *S. aureus* research. DNA microarray analysis of large samples of clinically characterised isolates provides broad insights into evolution, pathogenesis, and disease emergence.

1.6.10 Comparative studies on different typing methods

A number of DNA-based methods have therefore been developed for typing *S. aureus* strains. Pulsed-field gel electrophoresis (PFGE) analysis is an accurate, reliable, and discriminatory method used by many hospitals and reference laboratories (Bannerman *et al.,* 1995; Shimizu *et al.,* 1997a; 1997b; McCullagh *et al.,* 1998; Shimizu *et al.,* 2000; Zadoks *et al.,* 2000), but it is technically demanding and time-consuming. Compared to PFGE analysis, PCR-based typing methods offer the advantages of rapidity and simplicity. Repetitive-element PCR (REP-PCR) analysis based on multicopy elements of the staphylococcal genome has shown good reproducibility and discriminatory power in single-centre studies (Del Vecchio *et al.,* 1995; Cuny and Witte, 1996; Deplano *et al.,* 1997; Kumari *et al.,* 1997; van der Zee *et al.,* 1999; Deplano *et al.,* 2000). The MLST method was highly discriminatory and was validated by showing that pairs of human *S. aureus* isolates with the same allelic profile produced very similar *SmaI* restriction fragment patterns by PFGE. However, the major use of the MLST scheme for *S. aureus* will probably be to

unambiguously identify MRSA isolates and to probe the origins and evolution of these isolates (Enright *et al.*, 2000).

1.6.11 Identification and detection of *S. aureus* by PCR

Although *S. aureus* is easy to cultivate and is not difficult to identify, some PCR methods have been developed for rapid identification of *S. aureus*. Brakstad *et al.* (1992) developed a PCR assay specific for *S. aureus* by targeting the *nuc* gene coding for staphylococcal thermonuclease. This assay was shown to be suitable for diagnostic purposes. Martineau *et al.* (1998) described the development of another species-specific DNA-based assay for the identification of *S. aureus*. In ordered to select a genetic target suitable for diagnostic purposes, a *S. aureus* genomic library was screened by hybridization of DNAs from an array of both Gram-positive and Gram-negative bacterial species. Using this strategy, they were able to identify a clone carrying a chromosomal DNA insert suitable as a probe for the identification of *S. aureus*. The genomic DNA fragment inserted into this clone was species-specific and ubiquitous (i.e., it hybridized to DNA from any *S. aureus* strain) for the identification of *S. aureus*. This genomic DNA fragment was sequenced and was used to design PCR amplification primers for *S. aureus*-specific PCR assays. The simple and rapid PCR assay that they developed provided a new tool for the diagnosis of *S. aureus* infections, and the assay may be applied for the direct detection of *S. aureus* from blood culture or clinical specimens.

1.7 AIMS OF RESEARCH

Staphylococci are a common cause of infection in birds and most infection are caused by coagulase-positive staphylococci, especially *Staphylococcus aureus*. However much still remains to be done to complete our knowledge of *Staphylococcus aureus* from birds. There is very little information available on the molecular characterisation of avian *Staphylococcus aureus* strains and their virulence genes. The approach in this study was to obtain a number of isolates of avian *S. aureus*, which were mainly from diseased birds in commercial flocks from different geographic areas, and to characterise these strains by a variety of conventional and molecular methods. The aims were two-fold: to develop discriminating and reproducible identification and typing systems to investigate disease outbreaks, and to understand the disease potential of *S. aureus* in poultry. Any information gained would be of benefit to the poultry industry.

2. MATERIALS AND METHODS

2.1 BACTERIAL STRAINS

A total of 130 bacterial isolates were used. They belonged to different species of staphylococci and a description of each isolate is given in **Tables 2.1**, **2.2**, **2.3**, **2.4**, **2.5** and **2.6**. Identification of isolates at species level was clear, with isolates showing properties similar to those described by Kloos and Schleifer (1975), Kloos and Lambe (1991), Kloos (1998).

2.1.1 Avian coagulase-positive strains

The 87 isolates were obtained during the study (**Table 2.1**). The strains were field isolates and were from diseased birds. There was no type strain available in any of the major culture collections, but the strain selected as a control, strain 24, was obtained from a case of proximal femoral degeneration, in a broiler from Northern Ireland.

2.1.2 Avian coagulase-negative strains

The six isolates of three different coagulase-negative staphylococcal species were included as controls in the study (**Table 2.2**). There was no type strain available for these species in any of the culture collections. These bacteria were isolated from diseased flocks.

2.1.3 Human coagulase-positive strains

Twenty one human isolates were obtained for the study (**Table 2.3**) including the type strain of *S. aureus*.

2.1.4 Human coagulase-negative strains

Three isolates were used. They belonged to two species of bacteria, namely *S. epidermidis* and *S. saprophyticus* (**Table 2.4**).

2.1.5 Animal coagulase-positive strains

Eight isolates were obtained during the study (**Table 2.5**). They belonged to two

Table 2.1 Description of avian coagulase-positive isolates

Isolate[#]	Source	Site of isolate	Disease status	Geographic origin	Origin[†]
2A	Chicken	Joint	SYN[a]	Scotland	DJT
2B	Chicken	Joint	SYN	Scotland	DJT
3A	Chicken	Joint	FHN[b], TID[c], SYN	Scotland	DJT
4	Chicken	Joint	FHN, SYN RTI[d]	Scotland	DJT
5	Chicken	Gut	No record	Scotland	DJT
6	Chicken	Joint	FHN, SYN, TID, RTI	Scotland	DJT
7A	Chicken	Joint	SYN	Scotland	DJT
7B	Chicken	Joint	SYN	Scotland	DJT
8	Chicken	Joint	SYN	Scotland	DJT
31174	Chicken	Heart	MSH[e]	Scotland	DJT
31175	Chicken	Yolk sac	YSI[f]	Scotland	DJT
31176	Chicken	Heart	YSI	Scotland	DJT
31177	Chicken	Heart	MSH	Scotland	DJT
31178	Chicken	Heart	YSI	Scotland	DJT
31179	Chicken	Heart	YSI	Scotland	DJT
31180	Chicken	Heart	YSI	Scotland	DJT
31181	Chicken	Heart	YSI	Scotland	DJT
31182	Chicken	Heart	MSH/YSI	Scotland	DJT
31183	Chicken	Heart	MSH/YSI	Scotland	DJT
31036	Chicken	Skin	Skin scalp lesion	Scotland	DJT
31039	Chicken	Skin	Skin scalp lesion	Scotland	DJT
32528	Cockatoo	No record	No record	Scotland	DJT
E	Goshawk	Foot	Bumblefoot	England	NAF
K	Saker falcon	Foot	Bumblefoot	England	NAF
SV6	Steppes falcon	Foot	Bumblefoot	England	NAF
SV132	Harris Hawk	Foot	Bumblefoot	England	NAF
SV289	Lanner falcon	Foot	Bumblefoot	England	NAF
NFR	Gyr falcon	Foot	Bumblefoot	England	NAF
PD486	Chicken	Joint	SYN	Sri Lanka	SA
PD458	Chicken	Joint	SYN	Sri Lanka	SA
PD380	Chicken	Liver	Pericarditis	Sri Lanka	SA
PD27	Chicken	Liver	YSI	Sri Lanka	SA
199	Duck	No record	No record	USA	RJM
214	Duck	No record	No record	USA	RJM
217	Duck	No record	No record	USA	RJM
235	Duck	No record	No record	USA	RJM

Table 2.1 Continued...

Table 2.1 Continued...

Isolate	Source	Site of isolate	Disease status	Geographic origin	Origin
24*	Chicken	No record	Clinical In.[g]	N. Ireland	JJM
34	Chicken	No record	Clinical In.	N. Ireland	JJM
35	Chicken	No record	Clinical In.	N. Ireland	JJM
36	Chicken	No record	Clinical In.	N. Ireland	JJM
38	Chicken	No record	Clinical In.	N. Ireland	JJM
77	Chicken	No record	Clinical In.	N. Ireland	JJM
82	Chicken	No record	Clinical In.	N. Ireland	JJM
83	Chicken	No record	Clinical In.	N. Ireland	JJM
85	Chicken	No record	Clinical In.	N. Ireland	JJM
86	Chicken	No record	Clinical In.	N. Ireland	JJM
98	Chicken	No record	Clinical In.	N. Ireland	JJM
99	Chicken	No record	Clinical In.	N. Ireland	JJM
100	Chicken	No record	Clinical In.	N. Ireland	JJM
104	Chicken	No record	Clinical In.	N. Ireland	JJM
105	Chicken	No record	Clinical In.	N. Ireland	JJM
122	Chicken	No record	Clinical In.	N. Ireland	JJM
123	Chicken	No record	Clinical In.	N. Ireland	JJM
496	Chicken	No record	Clinical In.	N. Ireland	JJM
562	Chicken	No record	Clinical In.	N. Ireland	JJM
564	Chicken	No record	Clinical In.	N. Ireland	JJM
572	Chicken	No record	Clinical In.	N. Ireland	JJM
580	Chicken	No record	Clinical In.	N. Ireland	JJM
582	Chicken	No record	Clinical In.	N. Ireland	JJM
595	Chicken	No record	Clinical In.	N. Ireland	JJM
596	Chicken	No record	Clinical In.	N. Ireland	JJM
597	Chicken	No record	Clinical In.	N. Ireland	JJM
598	Chicken	No record	Clinical In.	N. Ireland	JJM
3	Chicken	No record	Clinical In.	N. Ireland	JJM
11	Chicken	No record	Clinical In.	N. Ireland	JJM
20	Chicken	No record	Clinical In.	N. Ireland	JJM
25	Chicken	No record	Clinical In.	N. Ireland	JJM
30	Chicken	No record	Clinical In.	N. Ireland	JJM
48	Chicken	No record	Clinical In.	N. Ireland	JJM
51	Chicken	No record	Clinical In.	N. Ireland	JJM
52	Chicken	No record	Clinical In.	N. Ireland	JJM
53	Chicken	No record	Clinical In.	N. Ireland	JJM
88	Chicken	No record	Clinical In.	N. Ireland	JJM
170	Chicken	No record	Clinical In.	N. Ireland	JJM
172	Chicken	No record	Clinical In.	N. Ireland	JJM
259	Chicken	No record	Clinical In.	N. Ireland	JJM
329	Chicken	No record	Clinical In.	N. Ireland	JJM
330	Chicken	No record	Clinical In.	N. Ireland	JJM

Table 2.1 Continued...

Table 2.1 Continued...

Isolate	Source	Site of isolate	Disease status	Geographic origin	Origin
352	Chicken	No record	Clinical In.	N. Ireland	JJM
373	Chicken	No record	Clinical In.	N. Ireland	JJM
390	Chicken	No record	Clinical In.	N. Ireland	JJM
410	Chicken	No record	Clinical In.	N. Ireland	JJM
451	Chicken	No record	Clinical In.	N. Ireland	JJM
31	Chicken	No record	Clinical In.	N. Ireland	JJM
33	Chicken	No record	Clinical In.	N. Ireland	JJM
47	Chicken	No record	Clinical In.	N. Ireland	JJM
525	Chicken	No record	Clinical In.	N. Ireland	JJM

[#]All isolates are *S. aureus* except for isolate SV132 which is *S. intermedius.*

[†]Origin, DJT: Prof. D. J. Taylor, Department of Veterinary Pathology, University of Glasgow; NAF: Dr. N. A. Forbes, Lansdown Veterinary Surgeons, The Clockhouse Veterinary Hospital, Wallbridge, Stroud, Gloucestershire; SA: Dr. S. Appuhamy, Veterinary Research Institute, Peradeniya, Sri Lanka; RJM: Dr. R. J. Montali, Department of Pathology, National Zoological Park, Washington DC, USA; JJM: Dr. J. J. McCullagh, Veterinary Science Division of Agriculture for Northern Ireland.

SYN[a]: Synovitis, FHN[b]: Femoral head necrosis; TID[c]: Tibial dischondroplasia; RTI[d]: Rotated tibia; MSH[e]: Mushy chick disease; YSI[f]: Yolk sac infection; Clinical In.[g]: Clinical infection.

24[*]: Strain 24, was obtained from a case of proximal femoral degeneration, in a broiler from Northern Ireland.

Table 2.2 Description of avian coagulase-negative isolates

Isolate	Source	Site of isolate	Disease status	Geographic origin	Origin[†]
31037[a]	Chicken	Skin	Skin lesion	Scotland	DJT
31038[b]	Chicken	Skin	Skin lesion	Scotland	DJT
31040[b]	Chicken	Skin	Skin lesion	Scotland	DJT
31041[b]	Chicken	Skin	Skin lesion	Scotland	DJT
31051[c]	Chicken	Skin	Skin lesion	Scotland	DJT
3B[a]	Chicken	Joint	FHN[d], TID[e], SYN[f]	Scotland	DJT

[†]DJT: Professor D. J. Taylor, Department of Veterinary Pathology, University of Glasgow,
[a]*S. cohnii*, [b]*S. sciuri*, [c]*S. caprae*,
FHN[d]: Femoral Head Necrosis, TID[e]: Tibial dischondroplasia and SYN[f]: Synovitis.

Table 2.3 Description of human *S. aureus* isolates

Isolate	Site of isolate	Disease status	Geographic origin	Origin[†]
HUM-2	No record	No record	Scotland	DP
HUM-3	No record	No record	Scotland	DP
CN4575	No record	No record	No record	THB
HUM-11	No record	No record	Scotland	DP
8530*	No record	No record	No record	NCTC
RN4220	No record	No record	No record	JM
HUM-108	Foot	No record	Scotland	DP
HUM-109	Foot	No record	Scotland	DP
HUM-G1	No record	No record	Scotland	CG
HUM-G2	No record	No record	Scotland	CG
HUM-G3	No record	No record	Scotland	CG
HUM-G4	No record	No record	Scotland	CG
HUM-G5	No record	No record	Scotland	CG
HUM-G6	No record	No record	Scotland	CG
HUM-271	No record	No record	N. Ireland	JJM
HUM-275	No record	No record	N. Ireland	JJM
HUM-282	No record	No record	N. Ireland	JJM
HUM-284	No record	No record	N. Ireland	JJM
HUM-294	No record	No record	N. Ireland	JJM
HUM-298	No record	No record	N. Ireland	JJM
HUM-BB	No record	No record	No record	THB

[†]DP: Dr. D. Platt, Department of Bacteriology, Glasgow Royal Infirmary; THB: Dr. T. H. Birkbeck, Division of Infection and Immunity, University of Glasgow; NCTC: National Collection of Type Culture, PHLS Central Public Health Laboratory, Colindale, London; JM: Dr. J. Murray, University of Sheffield; CG: Prof. C. Gemmell, Department of Bacteriology, Glasgow Royal Infirmary and JJM: Dr. J. J. McCullagh, Veterinary Science Division of Agriculture for Northern Ireland.

8530*: NCTC type strain.

species namely *S. aureus* and *S. intermedius* and were field isolates. There was no type strain available in any of the major culture collections.

2.1.6 Animal coagulase-negative strains

Five isolates of four species, namely *S. epidermidis*, *S. warneri*, *S. hominis* and *S. chromogenes* were included in the study (**Table 2.6**).

2.2 GENERAL BACTERIOLOGICAL PROCEDURES

2.2.1 Growth media

Tryptone soya agar (TSA) (Oxoid) 4% (w/v), Mannitol salt agar (MSA) (Oxoid) 11.1% (w/v), Blood agar base no. 2 (Oxoid) 4% (w/v) supplemented with defibrinated sheep blood (E and O Laboratories Ltd., Bonnybridge, UK) of, 5% (v/v) (SBA), Nutrient agar (NA) (Oxoid) 2.8% (w/v) and Luria Bertani broth (LB) (**Appendix I**) were used as solid media for routine growth of bacteria. Brain heart infusion broth (BHIB) (Oxoid) 3.7% (w/v) was used as liquid medium.

2.2.2 Sterilisation of culture media

All the culture media were sterilised by autoclaving at 15 lbs p.s.i (121 °C) for 15 min unless otherwise stated.

2.2.3 Storage of isolates

The isolates were received on swabs, agar slopes or in freeze-dried ampoules. They were subcultured onto TSA, MSA, SBA and NA and incubated as described in section 2.2.5. After assessing their purity (see below) they were harvested from MSA plates, suspended in BHIB plus sterile glycerol 10% (v/v) and stored as aliquots in sterile plastic vials at –70 °C. Subsequent cultures were made from these frozen stocks and used for all experiments.

Table 2.4 Description of human coagulase-negative isolates

Isolate	Site of isolate	Disease status	Geographic origin	Origin[†]
134[a]	No record	No record	No record	CG
1-TL[a]	No record	No record	No record	Teaching lab
132[b]	No record	No record	No record	CG

[†]CG: Prof. C. Gemmell, Department of Bacteriology, Glasgow Royal Infirmary; Teaching lab: The Teaching Laboratory, Division of Infection and Immunity, University of Glasgow.
[a]*S. epidermidis*
[b]*S. saprophyticus*

Table 2.5 Description of animal coagulase-positive isolates

Isolate	Source	Site of isolate	Disease status	Geographic origin	Origin[†]
HOR-31912[a]	Horse	Skin	No record	Scotland	DJT
DOG-32236[b]	Dog	Skin	No record	Scotland	DJT
BOV-32276[a]	Bovine	Milk	Mastitis	Scotland	DJT
RAB-32253[b]	Rabbit	Skin	No record	Scotland	DJT
ZM-7[a]	Zoo Mouse	No record	No record	No record	THB
BOV-65[a]	Bovine	No record	No record	N. Ireland	JJM
BOV-66[a]	Bovine	No record	No record	N. Ireland	JJM
BOV-67[a]	Bovine	No record	No record	N. Ireland	JJM

[†]DJT: Professor D. J. Taylor, Department of Veterinary Pathology, University of Glasgow, THB: Dr. T. H. Birkbeck; Division of Infection and Immunity, University of Glasgow, JJM: Dr. J. J. McCullagh, Veterinary Science Division of Agriculture for Northern Ireland.
[a]*S. aureus*
[b]*S. intermedius*

Table 2.6 Description of animal coagulase-negative isolates

Isolate	Source	Site of isolate	Disease status	Geographic origin	Origin[†]
DUK-31915[a]	Duck	Feathers	No record	Scotland	DJT
BOV-32265[b]	Bovine	Milk	Mastitis	Scotland	DJT
BOV-32268[c]	Bovine	Milk	Mastitis	Scotland	DJT
BOV-32298[d]	Bovine	Milk	Mastitis	Scotland	DJT
BOV-32304[d]	Bovine	Milk	Mastitis	Scotland	DJT

[†]DJT: Professor D. J. Taylor, Department of Veterinary Pathology, University of Glasgow.
[a]Isolate was *S. hominis*
[b]Isolate was *S. warneri*
[c]Isolate was *S. chromogenes*
[d]Isolate were *S. epidermidis*

2.2.4 Culture purity checks

All routine agar plates and broth cultures were subjected to thorough purity checks by Gram staining and by culture. The broth cultures were inoculated (100 µl) onto TSA plates and, after incubation, checked for purity of colony growth.

2.2.5 Incubation conditions

All cultures of staphylococci on MSA were incubated at 37 °C for 48 h and for other media at 37 °C for 24 h.

2.3 IDENTITY OF TEST ISOLATES

Isolates were received after identification in the source laboratories. To confirm their identity for this study, some of the isolates were subjected to cultural and biochemical tests. In addition, the identity of all isolates was determined by the API Staph and Rapidec Staph systems (bioMérieux, Marcy-l' Etoile, France).

2.3.1 Cultural and biochemical tests

Identity of the isolates was assessed according to the results of a panel of basic tests. These were Gram staining; colony morphology; production of coagulase; nuclease production; catalase and haemolysins; various enzyme activities and carbohydrate fermentation. These tests were performed according to the methods of Kloos and Lambe (1991) or were included in the API Staph and Rapidec Staph multi-test kits described below.

2.3.2 Biotyping

The 40 strains from N. Ireland were examined using a biotyping scheme described by Devriese (1984) and Isigidi *et al.* (1990) (**Table 2.7**) and results were provided by Dr. J. J. McCullagh, Veterinary Science Division of Agriculture for Northern Ireland, Belfast.

Table 2.7 ***Staphylococcus aureus*** **biotypes (Devriese, 1984; Isigidi *et al.*, 1990)**

Biotype	Staphylokinase	β-haemolysin	Bovine plasma coagulation within 6 h	Crystal violet type	Protein A
Human β-ve	+	-	-	C	*[a]
Human β+ve	+	+	-	C	*[a]
Slaughterhouse	-	-	-	A	+
Poultry	-	-	-	A	-
Bovine	-	+	+	A	*[a]
Ovine	-	+	+	C	*[a]

[a]Test result not required.

2.3.3 Rapidec Staph

Tests were carried out according to the manufacturer's instructions. Sterile distilled water 250 µl was added to cupules C and S. The level of the water was 1 mm below the top edge of the cupule. The contents of cupule C were gently homogenised with an applicator stick and then, with the end of a new stick, several colonies of the same morphology were picked from a culture plate and homogenised in cupule S until a turbidity equivalent to that in cupule C (McFarland 4) was obtained. 50 µl of the bacterial suspension (S) was transferred into each of cupules 0, 1, 2 and 3 by using a Pasteur pipette and then a lid was placed on the strip and the strip incubated for 2 hours at 37 °C. At the end of the incubation period, all cupules were examined for the fluorescent reaction, spontaneous coloured reaction, and the β-galatosidase and catalase tests were carried out according **Table 2.8**.

2.3.4 API Staph

Again, tests were carried out according to the manufacturer's instructions. An incubation box (tray and lid) was prepared by distributing about 5 ml of distilled water into the trays. The test organisms were culture on blood agar for 18-24 h at 37 °C and checked that they belonged to the *Micrococcaceae* family (by morphology, Gram stain, catalase etc.) and also that the culture was pure. An ampoule of API Staph Medium was opened and homogeneous bacterial suspension with a turbidity equal to 0.5 McFarland standard was prepared. The microtubes were then filled with the inoculated API Staph Medium. Anaerobiosis in the ADH and URE tests was ensured by filling the cupules with sterile mineral oil to form a convex meniscus. The trays were incubated at 37 °C for 18-24 h. Reactions were developed by adding 1 drop of each of the reagents, VP, NIT and ZYM to appropriate cupules and then the colour reactions obtained after 10 min were noted. The different tests and the colour grades obtained in the API Staph are detailed in **Table 2.9**.

Table 2.8 Reading table of Rapidec Staph for different species of staphylococci

Fluorescence control	Fluorescent reaction	Spontaneous reaction	Reaction to reveal	Results
Cupule 0	Cupule 1	Cupule 2	Cupule 3	
	[a]AUR (UV)	[b]PAL	[c]β GAL (FVB)	
	Fluorescent	Yellow or colourless	colourless	*S. aureus*
	colourless	yellow	pink/red	*S. intermedius*
	colourless	yellow	pink/red	*S. xylosus*
	colourless	yellow	colourless/yellow	*S. epidermidis*
	colourless	colourless	pink/red	*S. saprophyticus*
	colourless	colourless	colourless/yellow	*Staphylococcus* spp.

[a]AUR (UV) : "aurease" (AUR) enzyme specific for *S. aureus*
[b]PAL : alkaline phosphatase
[c]β GAL (FVB) : β-galactosidase

Table 2.9 API Staph reading table

Test	Substrate	Reaction/Enzyme	Reading the strip	
			Negative	Positive
0	No substrate	Negative control	red	-
GLU	D-Glucose	(Positive control)		
FRU	D-Fructrose			
MNE	D-Mannose			
MAL	Maltose	Acidification due to		
LAC	Lactose	carbohydrate utilisation	red	yellow
TRE	D-Trehalose			
MAN	D-Mannitol			
XLT	Xylitol			
MEL	D-Melibiose			
NIT	Potassium nitrate	Reduction of nitrate	NIT 1+NIT 2/10 min	
		to nitrite	colourless-light pink	Yellow
PAL	β-naphthyl-acid phosphate	Alkaline phosphatase	ZYM A+ZYM B/10 min	
			Yellow	Violet
VP	Sodium pyruvate	Acetyl-methyl-carbinol	VP 1+VP 2/10 min	
		production	colourless	violet-pink
RAF	Raffinose			
XYL	Xylose			
SAC	Sucrose	Acidification due to	red	yellow
MDG	α-methyl-D-glucoside	carbohydrate utilisation		
NAG	N-acetyl-glucosamine			
ADH	Arginine	Arginine dihydrolase	yellow	orange-red
URE	Urea	Urease	yellow	red-violet

2.4 CHARACTERISATION OF ISOLATES BY PLASMID PROFILES AND ANTIBIOTIC SENSITIVITY

2.4.1 Plasmid DNA extraction

Isolates were grown in BHIB as above, then 1.5 ml of culture was centrifuged at 4,000 x g for 3 min, the pellet washed with 1 ml SET buffer (sucrose 20%, 50 mM EDTA, 50 mM Tris, pH 8.0) and then centrifuged at 13,000 x g. Pellets were resuspended in 150 µl of SET buffer, 5 µl of RNase (10 mg/ml) (Sigma) the mixture vortexed before 15 µl of lysostaphin (5 mg/ml) (Sigma) was added and the mixture incubated at 37 °C for 30 min. The plasmid preparation was done with a QIA Prep Spin Plasmid Kit (QIAGEN Ltd, Sussex, UK). This method is normally used for Gram-negative organisms but in this study was modified for staphylococci by including a lysostaphin lysis step (as above). The method was then continued according to the manufacturer's protocol that included resuspending pelleted bacterial cells in 250 µl of Buffer P1, transfer to a microfuge tube, and lysis using 250 µl of Buffer P2 (alkali-detergent solution) for 5 min at room temperature. The macromolecules were then precipitated using 350 µl of Buffer N3 (chaotropic solution) and then centrifuged at 15,000 x g for 10 min (Biofuge, Rotor: HFA 14.2). The supernate was added to a QIAprep® column and centrifuged at 10,000 x *g* for 1 min. The flow-through was discarded and the column was then washed with 500 µl of Buffer PB (trace nuclease removal) and centrifuged at 10,000 x *g* for 1 min. The flow-through was again discarded and the final wash was performed with 750 µl of Buffer PE (containing ethanol) with a further centrifugation, as described previously. An additional centrifugation was performed following flow-through removal to ensure thorough removal of ethanol. Finally, the DNA was eluted by centrifugation after addition of 50 µl of sterile distilled water. Ten to fifteen microliter of plasmid DNA was analysed after electrophorosis in agarose type II-A (Sigma) 0.7% (w/v) in Tris-borate-EDTA buffer (pH 8.0) containing ethidium bromide 0.5 mg/ml (see section 2.6.6).

2.4.2 Determination of antibiotic sensitivity profiles

Antibiotic sensitivity profiles of plasmid-containing and plasmidless isolates were determined by the ATB ANTIBIOGRAM (bioMérieux). The ATB strips contain 18 antibiotics at one or two concentrations (c and C). The antibiotics were penicillin G (0.25 mg/l),oxacillin (2 mg/l), kanamycin (8-16 mg/l), tobramycin (4-8 mg/l), gentamicin (4-8 mg/l), tetracycline (4 mg/l), minocyclin (4 mg/l), erythromycin (1-4 mg/l), lincomycin (2-8 mg/l), pristinamycin (2 mg/l), fosfomycin (32 mg/l), nitrofurantoin (25-100 mg/l), pefloxacin (1-4 mg/l), rifampicin (0.25-16 mg/l), fusidic acid (2-16 mg/l), vancomycin (4 mg/l), teicoplanin (4 mg/l) and cotrimoxazole (2/38-8/152 mg/l). Bacterial suspensions were prepared with a turbidity equivalent to 0.5 McFarland standard and added to each cupule. Strips were incubated for 18-24 h at 37 °C and observed for turbidity (+) in each cupule by visual reading.

2.4.3 Electrotransformation

2.4.3.1 Bacterial strains

S. aureus RN4220 (originally from R. P. Novick, New York University Medical Centre, kindly supplied by Dr. J. Murray, University of Sheffield) is a mutant of strain NCTC 8325 that lacks restriction barriers. This strain and avian *S. aureus* isolate 6 were used as recipients in electroporation experiments.

2.4.3.2 Plasmid purification

Plasmid DNA was isolated by the QIA Prep Spin Plasmid Kit (QIAGEN) as described above and then a 4.4 kb plasmid was purified from avian isolate *S. aureus* 7A by the QIAquick Gel Extraction Kit Protocol (QIAGEN). First, the plasmid band of 4.4 kb was excised from an agarose gel with a clean scalpel. The gel slice was weighed and 3 volumes of Buffer QG were added to 1 volume of gel. This was incubated at 50 °C for 10 min (or until the gel slice had completely dissolved). To help dissolve the gel, the tube was mixed by vortexing every 2-3 min during the incubation. After the gel slice had dissolved

completely, the colour of the mixture was yellow (similar to Buffer QG without the dissolved agarose). One volume of isopropanol was then added to the gel sample and mixed. The mixture was then placed in a QIAquick spin column provided with a 2 ml collection tube. This was centrifuged for 1 min, the flow-through discarded and 0.5 ml of Buffer QG was then added to the QIAquick column and centrifuged for 1 min. To wash, 0.75 ml of Buffer PE was added and again the column was centrifuged for 1 min. The flow-through was discarded and the column centrifuged for an additional 1 min at ≥10,000 xg (~13,000 rpm). The column was then placed into a clean 1.5 ml microfuge tube. To elute the DNA, 50 µl of H_2O was added onto the centre of the QIAquick membrane, allowed to stand for 5 min (not 1 min. as in the protocol) and then the column was centrifuged for 1 min at maximum speed.

2.4.3.3 Solutions and reagents for electroporation

1. B2 broth: casein hydrolysate 10 g, yeast extract 25 g, glucose 5 g, NaCl 25 g, K_2HPO_4 1 g, dissolved in 1 litre of water. The pH was adjusted to 7.5 and then the medium was autoclaved.
2. 0.2 M sodium hydrogen maleate: maleic anhydride 11.6 g, NaOH 4 g dissolved in 500 ml water and then autoclaved.
3. 4 X Penassay broth: penassay medium (antibiotic medium 3; Difco Laboratories Limited, Surrey, UK) 17.5 g dissolved in 250 ml water and then autoclaved.
4. 2 X SMM: 25 ml of 0.2 M sodium hydrogen maleate, 40 ml of 0.1 M NaOH. pH adjusted to 6.5. 5 ml of 1 M $MgCl_2$ and 42.7 g sucrose were added to the mixture, dissolved and the final volume adjusted to 125 ml with water. This solution was filter-sterilised.
5. SMMP: 55 ml of 2 X SMM, pH 6.8, 40 ml of 4 X penassay broth, bovine serum albumin (Sigma) 10% (w/v). pH adjusted to 7.0 and filter-sterilised.
6. Glycerol 10% (v/v) in water and then filter-sterilised.

2.4.3.4 Preparation of electrocompetent cells

A single colony of a selected strain was inoculated into 3 ml of B2 broth and the culture incubated overnight at 37 °C with constant aeration. 1.5 ml of the overnight culture was added to 150 ml of fresh B2 broth in a 1 litre flask and incubated with constant aeration (~250 rpm) at 37 °C until the OD at 650 nm reached 0.5. The culture was then chilled on ice for 15 min to stop growth. The cells were harvested by centrifugation at 12,000 x g for 15 min at 4 °C, washed three times in an equal volume of sterile water and washed once with 30 ml of glycerol 10% (v/v). After centrifugation, the bacterial pellet was resuspended in 15 ml of glycerol 10% (v/v), and incubated at 20 °C for 15 min. The cell suspension was then centrifuged again as described above, and the cells suspended in 5 ml of glycerol 10% (v/v). The final cell concentration should be $\geq 1 \times 10^{10}$ colony-forming units (cfu)/ml. Aliquots (250 µl) of the electrocompetent cells were prepared in sterile 1.5 ml tubes. The cells were used fresh or stored after flash-freezing in dry ice. Electrocompetent staphylococci are stable for several months at –70 °C.

2.4.3.5 Electroporation procedure

Prior to electroporation, a 0.2 cm electroporation cuvette (Flowgen, UK) and the safety chamber were chilled at –20 °C. To the cold cuvette, 1-5 µl of DNA (ligation mix or plasmid preparation) and 40 µl of competent cells were added. The mixture was shaken to the bottom of the cuvette and a Bio-Rad Gene Pulser (model 1652078, Bio-Rad laboratories, UK) set at 2.5 KV, 25 µFD was connected to a Bio-Rad pulse controller (model 1652098) set to 100 Ω. The cells were pulsed once to give a time constant of 4-5 msec. Immediately following electroporation, the cells were resuspended in 950 µl of SMMP. The mixture was transferred to a sterile culture tube and incubated at 37 °C without shaking for 1 h. Aliquots of the electroporation mixture (50-100 µl) were spread on LB agar medium containing tetracycline (5 µg/ml) and plates incubated up to 48 h at 37 °C.

2.5 CHARACTERISATION OF EXOPROTEINS

2.5.1 Expression of exoproteins

A single colony of an isolate was inoculated into 5 ml of BHI broth and shaken at 37 °C overnight. The culture was then centrifuged at 5,000 x g for 5 min and then supernates were filter-sterilised with a 0.22 μm pore size filter. Supernates were then concentrated 30x at 14,000 xg for 30 min in a Microcon Centrifugal Filter Device model YM-30 (pore size 30 kDa) (Millipore, USA).

2.5.2 Sample preparation for electrophoresis

The samples were made up to a total volume of 50 μl with 8 μl of 6x protein loading dye (**Appendix I**) was added. Samples were stored at –20 °C until loading. Immediately before use, samples were boiled in a water bath for 10 min. A 10 kDa protein ladder (Gibco BRL) or Unstained Protein Ladder (Bio-Rad) were used according to the manufacturer's instructions.

2.5.3 SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970) in a vertical electrophoresis gel tank (model SE600, Hoefer Scientific Instruments, California, USA). The composition of all solutions can be found in **Appendix I**. Glass plates were assembled according to the manufacturer's instructions. Resolving gels, containing acrylamide 12% (w/v), were poured between the assembled plates until the gel was 4 cm below the top of the plates and ethanol 100% (v/v) was used as an overlay. The gel was left undisturbed for approximately 1 h at room temperature until set. The ethanol was poured off and the gel surface washed with distilled water. A stacking gel containing acrylamide 6% (w/v) was poured onto the polymerised separating gel and a comb was placed into the gel solution. Again the gel was left for approximately 1 h at room temperature and the comb removed. The wells were immediately filled with 1x electrode buffer and assembled into the electrophoresis gel tank.

Samples were added to the wells. The lower buffer reservoir was stirred with a magnetic stirrer during vertical gel electrophoresis. The gel was run at 20 mA until the dye entered the separating (lower) gel when the current was increased to 30 mA. The gel was removed when the tracker dye reached the bottom of the separating gel (approximately 3 h). The gel was stained or blotted as appropriate. For staining, Coomassie blue stain (**Appendix I**) was utilised for a minimum of 2 h on a rotating platform. The stain was then replaced with destain solution until the background was decolourised.

2.5.4 Skim milk agar plates

Skim milk agar plates were used in order to examine the protease activity of the isolates. Briefly, the isolates to be tested were inoculated into Tryptone soy agar (Oxoid) containing skim milk 2% (w/v). After incubation for 24 h at 37 °C, the presence of a clear zone around a colony was judged as a positive reaction for protease.

2.5.5 Effect of different reagents on protease activity

The effect of different reagents on protease activity was measured using azocasein (Sigma) as a substrate. Briefly, 100 µl of cultural supernate was activated or inhibited by individual incubation with 50 mM L-cysteine (cysteine protease activator) (Sigma), 1.3 mM EDTA-Na_2 (metalloprotease inhibitor) (Fisher Scientific, UK), 1 µM pepstatin (aspartic acid protease inhibitor) (Sigma), 20 µM APMSF [(4-amidino-phenyl) methane-sulfonyl fluoride] (serine protease inhibitor) (Sigma), 130 µM bestatin (amino peptidase inhibitor) and 28 µM E64 (N-(N-(L-3-trans-carboxirane-2-carbonyl)-L-leucyl)-agmatine) (cysteine protease inhibitor) (Sigma) at 37 °C for 30 min. Then, 400 µl of azocasein (2 mg/ml) in 50 mM Tris-HCl buffer, pH 7.2 was added to the activated or inhibited protease solutions and they were incubated at 37 °C for 2 h. The reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid (TCA) (BDH Laboratory Supplies, UK). After centrifugation, 1 ml of the

supernatant was mixed with the same volume of 0.5 M NaOH and the optical density was determined at 440 nm.

2.5.6 Protease analysis on SDS-PAGE gelatin gels

Proteases were analysed by non-reducing SDS-PAGE (12% (w/v) acrylamide) by using Coomassie blue stain to detect the hydrolysis of gelatin co-polymerised in the gel as previously described (Lockwood, *et al.*, 1987). After electrophoresis, the gel was removed from the plates and then was washed free of SDS in 2.5% (v/v) Triton X-100 solution at 37 °C for 30 min. Gelatin hydrolysis was performed at 37 °C in 0.1 M sodium acetate buffer pH 9.0 containing 1 mM DTT for 2 h. For staining, Coomassie blue stain (**Appendix I**) was utilised for a minimum of 2 h on a rotating platform. The stain was then replaced with destain solution until the background was decolourised.

2.5.7 Determination of N-terminal amino acid sequence of 22 kDa protein

Concentrated culture supernates were subjected to SDS-PAGE as above (see section 2.5.3) and bands were transferred electrophoretically onto a PVDF membrane (Bio-Rad) by the method of Matsudaira (1987). After staining with Coomassie brilliant blue R-250, a major protein band of interest (22 kDa) was cut out and sent to the Protein and Nucleic Acid Chemistry Laboratory (PNACL) at the University of Leicester for N-terminal amino acid sequence determination.

2.5.8 Amplification of N-terminal sequence of 22 kDa protein

Template DNA used for this amplification was prepared according to Section 2.6.1. After sequencing of the N-terminus of the 22 kDa protein, primers PRS-1 and PRS-2 were designed according to this sequence (**Table 2.11**). PCR amplification of the N-terminal sequence was carried out in 25 µl reaction volumes with Ready-To-Go Beads (Amersham Pharmacia biotech, UK). Each reaction mixture contained 1.5 unit of *Taq* DNA polymerase, 10 mM Tris-HCl, (pH 9.0 at room temperature), 50 mM KCl, 2.5 mM $MgCl_2$, 200 mm of each

dNTP, 100 pM of primer, 1 µl of 1 in 20 dilution of template DNA preparation and 16 µl of distilled water (double-processed tissue culture water) (Sigma). Amplification was done in a thermocycler as described in Section 2.6.4 by predenaturation at 94 °C for 4 min and then 35 cycles of denaturation at 94 °C for 30s, annealing at 50 °C for 45s and extension at 72 °C for 10s. Ten microlitres of amplified products were electrophoresed in agarose 3% (w/v) according to Section 2.6.5.

2.5.9 Southern blotting

All DNA-DNA hybridisations were detected non-radioactively using the Boehringer-Mannheim DIG-system kit, essentially as described in the "DIG System User's Guide" (van Miltenburg *et al.*, 1995).

2.5.9.1 Digestion of genomic DNA

Genomic DNA was purified according to the QIAGEN Genomic DNA (Genomic-tip 20/G) protocol (Cat. No. 19060) (QIAGEN Ltd, West Sussex, UK) (see section 2.6.1), for avian *S. aureus* (strains 31179 and 24) and human *S. aureus* (strains RN4220 and 8530) and six restriction digests (with *EcoRI*, *XbaI*, *SalI*, *RsaI*, *BamHI* and *HindIII*) were set up separately using the following protocol:

Genomic DNA	10 µl
Restriction enzyme (10 U/µl)	2 µl
Restriction enzyme buffer	3 µl
Deionised distilled H_2O	15 µl
Total	**30 µl**

The above were mixed gently and incubated at 37 °C for 16-18 h. Agarose gel electrophoresis was performed with 10 µl of each reaction mixture to examine for digestion.

2.5.9.2 Solutions

Southern blotting, DNA probe preparation, hybridisation and detection were performed with the reagents detailed in **Table 2.10**.

2.5.9.3 Probe labelling

The required probes were labelled using an intra-PCR DIG-labelling method. The standard PCR dNTP mixture (section 2.5.8) was replaced with PCR DIG labelling mix (Boehringer Mannheim). This labelling mix has a reduced amount of dTTP (1.9 mM) supplemented with 0.1 mM Dig-11-dUTP (digoxigenin linked to dUTP with an 11-atom spacer arm). The DIG labelled dUTP molecules are incorporated into the amplified DNA during the PCR reaction leading to a highly DIG-labelled probe DNA. The DIG-labelled DNA probes were purified to homogeneity using agarose gel electrophoresis, where the degree of label incorporated can be assessed by comparing the migration of the probe to unlabelled PCR product. The incorporated DIG label increases the mass of the probe DNA that can lead to low yields of DNA in purification using Silica based Qiaex II kit (Cat. No. 20051) (QIAGEN Ltd, West Sussex, UK). Therefore the probe was excised from the agarose and boiled in detection buffer (see **Table 2.10**) prior to hybridisation.

2.5.9.4 Blotting via capillary action

A agarose 0.8% (w/v) gel containing restriction digests of the appropriate chromosome samples was depurinated in 0.25 M HCl for 15 min. After rinsing in water the DNA samples were denatured for 30 min in 0.5 M NaOH, 1.5 M NaCl. The gel was rinsed in distilled water and neutralised by two 15 min washes (with shaking) in 0.5 M Tris-HCl, 3 M NaCl.

A raised gel support was placed into a dish and covered with a filter paper (Whatmann 3 MM) wick. The filter paper was saturated with 20x SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0). This buffer was also used to fill the reservoir around the raised gel support. The gel was placed onto the wick and

Table 2.10 Solutions for Southern blotting

Solution	Reagents
[a]dNTP DIG labelling mixture (10x)	2 mM dATP 2 mM dCTP 2 mM dGTP 1.9 mM dTTP 0.1 mM DIG-11-dUTP pH 7.0
20 x SSC	3 M NaCl 300 mM sodium citrate, pH 7.0
Southern Buffer 1	150 mM NaCl 100 mM maleic acid, pH7.5
[a]Southern Buffer 2	1% (w/v) nucleic acid blocking reagent in Buffer 1
Southern Buffer 3	100 mM Tris-HCl, pH 9.5 100 mM NaCl 50 mM $MgCl_2$
[a]NBT solution	92 mM (75 mg/ml) nitroblue tetrazolium salt in 70% (v/v) dimethylformamide
[a]X-Phosphate, 4-toluidine salt	115 mM (50 mg/ml) 5-bromo-4-chloro-3- indoyl phosphate 4-toluidine salt in dimethylformamide
Prehybridisation solution In 5x SSC was added:	N-laurylsarcosine 0.1% (v/v) SDS 0.02% (v/v) [a]Blocking reagent 1%(v/v)
Detection Buffer	0.1 Tris-HCl, pH 9.5 0.1 M NaCl

[a]All reagents were obtained from Boehringer Mannheim, Germany.

bubbles were removed by using a glass rod. A piece of positively-charged nylon membrane (Hybond N^+, Amersham International, Amersham, UK) cut to a size slightly larger than the gel and soaked in 20x SSC was placed on the gel. Three sheets of 3 MM paper (pre-soaked in 20x SSC) were placed on top of the membrane. A stack of paper towels cut to the size of the gel was placed on top of the 3 MM paper and a weight of approximately 1 kg was placed onto the paper towels. The blot apparatus was wrapped in cling-film and left overnight. The apparatus was dismantled and the DNA markers were identified (on UV transilluminator Model TM-40, UVP Inc., San Gabriel, California, USA) on the blot using a pencil (see section 2.6.6). The membrane was washed in 2x SSC at room temperature (1 min), wrapped in cling film and UV crossed linked (3 min on each side on UV transilluminator).

2.5.9.5 Prehybridisation and hybridisation

The membrane was prehybridised in a rolling hybridisation oven (model FH1DE, Techne Ltd, Duxford Cambridge, UK) for 2-3 h with 20 ml of prehybridisation solution at 65 °C. The probe was hybridised with the blot by incubating at 65 °C for 6-18 h in the hybridisation oven before removing the probe. After hybridisation, probes were saved and stored at –20 °C for future re-use. Excess probe was washed off the blot with two 5 min washes in 2 x SSC/SDS 0.1% (w/v). Two low stringency washes of 15 min at 65 °C, using 2 x SSC/SDS 0.1% (w/v), were followed by two higher stringency washes of 2x 15 min at 65 °C, using 0.1x SSC/SDS 0.1% (w/v).

2.5.9.6 Detection

The filter was washed briefly in Southern Buffer 1 (see **Table 2.10**) and incubated with Southern Buffer 2 (see **Table 2.10**) for 30 min (blocking). 1 µl of anti-digoxigenin (ovine F_{ab}) conjugated to alkaline phosphatase (Boehringer Mannheim, Germany) was added in 5 ml of Southern Buffer 1 and incubated with the membrane for 30 min. Two 15 min washes with Southern Buffer 1 removed excess antibody, followed by a 5 min wash in Southern Buffer 3 to

prepare the membrane for colour development. A volume of NBT solution (Boehringer Mannheim) (45 µl) and 35 µl X-phosphate solution (Boehringer Mannheim) in 10 ml Southern Buffer 3 were added, and colour allowed to develop in the dark. The reaction was stopped by washing the membrane in TE (10 mM Tris-HCl, pH, 7.6, 1 mM EDTA, pH, 8.0), and allowing the membrane to dry.

2.5.9.7 Amplification of cysteine protease gene of S. epidermidis

N-terminal sequence data was obtained for the 22 kDa protein found in the culture supernate of avian *S. aureus* strain 31179. No match was found in any of the *S. aureus* protein databases but, very recently, this sequence was matched in the protein database (NCBI) with an extracellular cysteine proteinase from *S. epidermidis* isolate 6746 (accession number AJ298299). Using this sequence, primers SACP-1 and SACP-2 were designed in this study (**Table 2.11**). DNA was prepared for this PCR amplification as described section 2.6.1. The reaction mixtures (50 µl) contained 45 µl of master mix; 1.25 units *Taq* DNA polymerase, 75 mM Tris-HCl (pH 8.8 at 25 °C), 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.01% (v/v) Tween 20, 0.2 mM of each dNTP (AB gene, Surrey, UK), for *S. aureus* isolates plus 1.0 mM of $MgCl_2$ (final concentration of $MgCl_2$ was 2.5 mM) for *S. aureus* and 1.5 mM ($MgCl_2$) for *S. epidermidis* isolates, 50 pM of each primers, 1 µl of template DNA preparation (diluted 1 in 20). All amplifications were accomplished by predenaturing at 94 °C for 4 min followed by 30 cycles of 30s for denaturing, 30s at 52 °C for annealing and 1 min at 72 °C for extension step followed by single cycle of 72 °C for 5 min. The PCR products were separated by electrophoresis of 15 µl of the reaction mixtures in ethidium bromide-stained agarose 1.2% (w/v) gels as described in Section 2.6.6.

2.6 CHARACTERISATION OF ISOLATES BY PCR METHODS

The main objective of this study was to identify and characterise the test isolates by PCR-fingerprinting and to characterise strains by their virulence-related genes.

2.6.1 Preparation of template DNA

Chromosomal DNA was extracted by a modification of the QIAGEN Genomic DNA (Cat. No. 19060) (QIAGEN) method. The 18 h liquid cultures (10 ml) of staphylococci in BHI broth were harvested by centrifugation at 15,000 x g for 5 min and the pellet resuspended in 3.5 ml of buffer B1 containing 50 µg/ml RNase A (Sigma) (i.e. 17.5 µl of 10 mg/ml). The mixture was vortexed to give a homogenous suspension and then 17.5 µl of lysostaphin stock (Sigma) solution (5 mg/ml, final concentration 25 µg/ml) was added and incubated for 15 min at 37 °C. The suspension cleared, although it remained slightly cloudy. It may be incubated longer if required. Protease stock solution (pronase 20 mg/ml) (Sigma), 100 µl, was then added and the mixture incubated at 37 °C for a further 15 min (it should go totally clear at this stage). Buffer B2, 1.2 ml was then added and the mixture vortexed for several seconds to mix thoroughly. The mixture was then incubated at 50 °C for 2 h. Finally 1 µl aliquots of the mixture were diluted with 19 µl of distilled water and stored at -20 °C.

2.6.2 Primers for PCR fingerprinting

The primers for the PCR were obtained from Gibco BRL, Paisley, UK. Their sequences are listed in **Table 2.11**. They were dissolved in sterile distilled water to give a final concentration of 50 pmol/µl. Schematic representation of the amplification of some of the different region of the *S. aureus* genome are shown in **Appendix II**.

2.6.3 Optimisation of PCR

As PCR is a highly sensitive technique, its performance may be adversely affected by slight variation of the components of the reaction mixture and the

Table 2.11 Primers used in PCR methods

PCR	Primer	Sequence[a] (5′-3′)	Reference
PCR-ribotyping	GIRRN	**GAAGTCGTAACAAGG**	Jensen *et al.* (1993)
	LIRRN	**CAAGGCATCCACCGT**	
PCR-ribotyping	STAA-AVI	**TCTTCAGAAGATGCG GAATA**	Forsman *et al.* (1997)
	STAA-AVII	**TAAGTCAAACGTTAA CATACG**	
REP-PCR	RW3A	**TCGCTCAAAACAACG ACACC**	DelVecchio *et al.* (1995)
Coagulase gene	COAG2	**CGAGACCAAGATTCA ACAAG**	Goh *et al.* (1992)
	COAG3	**AAAGAAAACCACTCA CATCA**	
Protein A gene	PROTA1	**TGTAAAACGACGGCC AGTGCTAAAAAGCTA AACGATGC**	Frenay *et al.* (1994)
	PROTA2	**CAGGAAACAGCTATGA CCCCACCAAATACAG TTGTTACC**	
Nuclease gene	NUC1	**GCGATTGATGGTGAT ACGGTT**	Brakstad *et al.* (1992)
	NUC2	**AGCCAAGCCTTGACG AACTAAAGC**	
agrP$_2$	AGR-1	**ATCAACTATTTTCCA TCACATCT**	Kornblum *et al.* (1990)
	AGR-2	**TTACACCACTCTCCT CACT**	
agrBDC	AGRD123F	**TATGCACCTGCAGCT ACTAA**	[†] Personal communication
	AGRD123R	**TCATGACGGAACTTG CGC**	

Table 2.11 Continued...

Table 2.11 Continued...

PCR	Primer	Sequence[a] (5′-3′)	Reference
sarA	SAR-1	**GCAATTACAAAAATC AATGATTGC**	Chien *et al.* (1998)
	SAR-2	**TTATAGTTCAATTTC GTTGTTTGC**	
entA[b]	SEA-1	**TGGGAAACGGTTAAA ACGAA**	Johnson *et al.* (1991)
	SEA-2	**GAACCTTCCCATCAA AAACA**	
entB[c]	SEB-3	**GTATGGTGGTGTAAC TGAGC**	*Personal communication
	SEB-4	**CCAAATAGTGACGAG TTAGA**	
entC[d]	SEC-3	**CTCAAGAACTAGACA TAAAAGCTAGG**	Becker *et al.* (1998)
	SEC-4	**TCAAAATCGGATTAA CATTATCC**	
entD[e]	SED-3	**CTAGTTTGGTAATAT CTCCTTTAAACG**	Becker *et al.* (1998)
	SED-4	**TTAATGCTATATCTT ATAGGGTAAACATC**	
entE[f]	SEE-3	**CAGTACCTATAGATA AAGTTAAAACAAGC**	Becker *et al.* (1998)
	SEE-2	**TAACTTACCGTGGAC CCTTC**	Johnson *et al.* (1991)
eta[g]	ETA-1	**CTATTTACTGTAGGA GCTAG**	Sakurai *et al.* (1995)
	ETA-2	**ATTTATTTGATGCTC TCTAT**	
etb[h]	ETB-1	**ATACACACATTACGG ATAAT**	Sakurai *et al.* (1995)
	ETB-2	**CAAAGTGTCTCCAAA AGTAT**	

Table 2.11 Continued

Table 2.11 Continued...

PCR	Primer	Sequence[a] (5′-3′)	Reference
tsst-1[i]	TST-3	**AAGCCCTTTGTTGCT TGCG**	Becker *et al.* (1998)
	TST-6	**ATCGAACTTTGGCCC ATACTTT**	
hla[j]	HLA-1	**GATATTAATATTAAA ACCGG**	This study
	HLA-2	**TTCTGAAGAACGATC TGTCC**	
hlb[k]	HLB-1	**ACTTAAATGTCATAA CAAC**	This study
	HLB-2	**CGTCCTTTTAGAACG AAGCG**	
hlg[l]	HLG-1	**AGCTCAACTTTATTA ACTTC**	This study
	HLG-2	**CTTACAGATAAAGTT GTAGC**	
hld[m]	HLD-1	**TTTAGTACTATACGA AGAT**	Janzon *et al.* (1989)
	HLD-2	**CCCTAATCGTACTTG C**	
16S rRNA	C70	**AGAGTTTGATYMTGG C**	Davies *et al.* (1996)
	C72	**GYTACCTTGTTACGA CTT**	
R.I.S.a[n]	SA442-1	**AATCTTTGTCGGTAC ACGATATTCTTCACG**	Martineau *et al.* (1998)
	SA442-2	**CGTAATGAGATTTCA GTAGATAATACAACA**	
N-terminal sequence of 22kDa band	PRS-1	**GTIMGIGCNCARTAY**	This study
	PRS-2	**YTGIGTYTCICKDAT**	

Table 2.11 Continued...

Table 2.11 Continued...

PCR	Primer	Sequence[a] (5′-3′)	Reference
Cysteine protease	SACP-1	**ATGMGAGCACAATAT GTAAATCAATTAAAG**	This study
	SACP-2	**ATAACCATAAATTGA TGCATACCATTCATA GTG**	

[a]A: adenine; T: thymine; C: cytosine; G: guanine; I: inosine; M: A+C; N: A+C+T+G; R: A+G; Y: C+T; K: T+G; D: A+T+G. [b]entA: enterotoxin A; [c]entB: enterotoxin B; [d]entC, enterotoxin C, [e]entD, enterotoxin D, [f]entE, enterotoxin E, [g]eta, exfoliatin A, [h]etb: exfoliatin B; [i]tsst-1: toxic shock syndrome toxin-1; [j]hla: α-haemolysin (α-toxin); [k]hlb: β-haemolysin (β-toxin); [l]hlg: γ-haemolysin (γ-toxin); [m]hld: δ-haemolysin (δ-toxin); [n]R.I.S.a: Rapid Identification of *S. aureus*; [†]Personal communication: Dr. Phil McDowell, Institute of Infections and Immunity, The University of Nottingham, Queens Medical Centre, Nottingham; *Personal communication: Prof. C. Gemmell, Department of Bacteriology, Glasgow Royal Infirmary.

reaction conditions. Each new application of PCR may require optimisation. Inadequate functioning of PCR may be observed as no detectable product or as a low yield of the target band, as high level of background due to mispriming or misextension of the formation of the primers or by the formation of primer-dimers. The concentration of the polymerase enzyme, deoxynucleotide triphosphate and magnesium ions are reported as critical components to be optimised in the PCR reaction (Innis and Gelfand, 1990). A simple procedure for optimisation of PCR based on orthogonal arrays was applied to avoid laborious steps (Cobb and Clarkson, 1994).

2.6.4 Components of the PCR

The components of the PCR were different for each specific amplification. Before setting the components for each PCR, different concentration of $MgCl_2$, primers and template DNA were explored and the optimum concentrations selected for each PCR.

2.6.5 Conditions for PCR

Amplification was done in a thermocycler (Touch Down, Hybaid Ltd, UK). The conditions were different for each PCR method. Different denaturing and annealing conditions were used and the best conditions finally selected.

2.6.6 Agarose gel electrophoresis

The amplified products (10-15 µl) were mixed with 1/6x volume of loading buffer (sucrose (BDH) 40% (w/v), bromophenol blue (Sigma) 0.25% (w/v)) to a final dilution of 1x and electrophoresed in agarose type II-A medium EEO (Sigma) 0.7-3% (w/v) in 0.5 x Tris-borate EDTA buffer (Tris base 89 mM, boric acid 89 mM, EDTA 2 mM, pH 8.0) containing ethidium bromide 0.5 µg/ml (BioRad, UK) (Sambrook *et al.,* 1989) in a horizontal submarine electrophoresis apparatus (E-C Apparatus Corporation, USA). The 1 kb, X174 RF DNA/HaeIII fragments and 25 bp ladders (GibcoBRL) were used as DNA molecular weight markers. The amplimers were visualised and photographed under UV light on a

transilluminator (Model TM-40, UVP Inc., San Gabriel, California, USA). The photographs were captured and scanned with Fotolook (version 2.07.2 Agfa, UK) using a scanner Studioscan *IIsi,* Agfa, or documented using Ultra Violet Products Gel Documentation System-Image Store 5,000, Version 7.2 (Ultra Violet Products Ltd., Cambrige) 2.5.1 and the labelling of images was done with Claris Draw Version 7.5.1. Photographs were inspected visually and different band profiles were given a number or letter whenever a distinct pattern was observed.

2.6.7 Gel extraction procedure

DNA was purified from agarose gels using the Qiaex®II purification kit (QIAGEN) according to manufacturer's instruction. The band of interest was excised from the gel and solubilised in Buffer QX1 (usually 3 volumes of buffer to one volume of gel). Qiaex resin (10-15 µl) was introduced and the mixture incubated at 50 °C for 10 min. Following centrifugation for 1 min at 10,000 x *g* the supernate was removed. The pellet was then washed once with 500 µl of Buffer QX1 and twice with 500 µl of Buffer PE, with centrifugation as before. The resin/DNA pellet was air-dried, 5-25 µl of sterile distilled water was added and, after incubation for 5 min at 50 °C, the resin was removed by centrifugation as before and the eluted DNA was retained

2.6.8 Prevention of contamination of DNA and decontamination

In order to minimise the cross contamination of reagents, sample etc. and false positive results, the guidelines suggested by Kwok and Higuchi (1989) were followed. All equipment such as micropipettes (Models P2, P10, P20, P200, P1000, P5000, Anachem Ltd., Luton, Beds, UK) and tips, and different areas in the laboratory were dedicated to the different stages of sample preparation, sample addition, setting up of PCR reactions, amplification and product detection.

All samples, reagents and amplified products were stored in assigned boxes in

separate -20 °C freezers in aliquots. The double-processed tissue culture water (distilled water) for the reaction mixture was prepared by filtration through a 0.22 μm filter (Millipore S. A., Molshiem, France) before autoclaving with single-use plastic materials and containers. All microfuge tubes and pipette tips were autoclaved to avoid the risk of nuclease activity. All the PCR experiments included negative controls without added DNA.

2.6.9 Molecular typing

2.6.9.1 PCR-ribotyping

The primers used were two different sets: GIRRN and LIRRN for method 1; STAA-AVI and STAA-AVII for method 2 (**Table 2.11**). The reaction mixture (50 μl) contained 45 μl of master mix (AB gene, Surrey, UK). 50 pM of each primer, 1 μl of template DNA preparation (diluted 1 in 20) and 2 μl of distilled water (double-processed tissue culture water). Amplifications were 30 cycles of: denaturation at 94 °C for 15s; annealing at 55 °C for 30s; extension at 72 °C for 1 min and 30s and then extension at 72 °C for 20 min. Fifteen microlitres of amplified products were analysed by electrophoresis in 1.2% agarose.

2.6.9.2 REP-PCR

The primer used was RW3A (**Table 2.11**). The REP-PCR was carried out in 25 μl reaction volumes with Ready-To-Go Beads (Amersham Pharmacia biotech, UK). Each reaction mixture contained 1.5 unit of *Taq* DNA polymerase, 10 mM Tris-HCl, (pH 9.0 at room temprature), 50 mM KCl, 1.5 mM $MgCl_2$, 200 mm of each dNTP, 75 pM of primer, 1 μl of 1 in 20 diluttion of template DNA preparation and 22.5 μl of distilled water (double-processed tissue culture water). Amplification was done in a thermocycler as above by predenaturation at 94 °C for 3 min and then 30 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min and extension at 72 °C for 2 min followed by a final extension at 72 °C for 5 min. Ten microlitres of amplified product were electophoresed in an agarose 1.2% (w/v) gels as above.

2.6.9.3 PCR for coagulase, protein A and nuclease genes

The primers used were COAG2 and COAG3 for the coagulase gene, PROTA1 and PROTA2 for X-region of the protein A gene and NUC1 and NUC2 for the nuclease region (**Table 2.11**). The reaction mixture (50 µl) for all of them contained 45 µl of master mix (AB gene, Surrey, UK), plus 1.0 mM of $MgCl_2$ (final concentration of $MgCl_2$ was 2.5 mM), 50 pM of each primers, 1 µl of template DNA preparation (diluted 1 in 20). All amplifications were accomplished by predenaturing at 95 °C for 4 min followed by 30 cycles of 30s for denaturing, 30s at 55 °C for annealing and 1 min at 72 °C for the extension step followed by a single extension at 72 °C for 5 min. The PCR products 15 µl were separated by electrophoresis as above.

2.6.9.4 PCR for 16S rRNA gene

Some avian and human *S. aureus* isolates were selected (**Table 2.12**) for sequence analysis of this gene. The primers used were C70 and C72 (**Table 2.11**). The reaction mixture (50 µl) contained 45 µl of master mix (AB gene, Surrey, UK), plus 1.0 mM of $MgCl_2$ (final concentration of $MgCl_2$ was 2.5mM), 50 pM of each primers, and 1 µl of template DNA preparation (diluted 1 in 20). All amplifications were accomplished by predenaturing at 94 °C for 10 min followed by 30 cycles of 45s at 94 °C for denaturing, 45s at 50 °C for annealing and 45s at 72 °C and a final extension step of 72 °C for 10 min. The PCR products 15 µl were separated by electrophoresis of 15 µl agarose 1.2% (w/v) gels as above.

2.6.9.5 Identification of S. aureus by PCR

The primers used were Sa442-1 and Sa442-2 (**Table 2.11**). The reactions mixture (50 µl) contained 45 µl of master mix (AB gene, Surrey, UK), plus 0.5 mM of $MgCl_2$ (final concentration of $MgCl_2$ was 2.0 mM), 50 pM of each primers, 1 µl of template DNA preparation (diluted 1 in 20). The PCR mixtures were subjected to thermal cycling for 3 min at 96 °C and then 30 cycles of 1s at 95 °C for the denaturation step and 30s at 55 °C for the annealing-extension step.

Fifteen microlitres of the PCR-amplified reaction mixture was resolved by electrophoresis through an agarose 2% (w/v) gel as above.

2.7 CHARACTERISATION OF VIRULENCE REGULATORY GENES BY PCR

2.7.1 *agrP2, agrBDC* and *sarA*

The primers used were AGR-1 and AGR-2 for *agrP2*, AGRD123F and AGRD123R for *agrBDC,* SAR-1 and SAR-2 for *sarA* (**Table 2.11**). Three separate PCRs were carried out in 50 µl reaction volume. Each reaction mixture contained 45 µl of master mix (AB gene, Surrey, UK), plus 1.0 mM of $MgCl_2$ (final concentration of $MgCl_2$ was 2.5 mM), 50 pM of each primers, 1 µl of template DNA preparation (diluted 1 in 20). Amplifications were 30 cycles of 1 min at 95 °C for denaturing, 1 min at 55 °C for annealing and 2 min at 72 °C for extension. Fifteen microlitres of each amplified product were electrophoresed in an 1.2% (w/v) agarose gels as above.

2.8 RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS

The 20 µl of reaction mixture contained 10 µl of purified PCR product (section 2.6.7), 1 µl (10 units) of restriction enzyme (New England Biolabs Ltd., Hertfordshire, UK), 5 µl of 1 x NEB Buffer 2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9 at 25 °C) and 4 µl of distilled water (double-processed tissue culture water). The reaction conditions were 37 °C in a heated block for 16 h. The sizes of the resulting bands were assessed after running the digest in an agarose 3% (w/v) gel with ethidium bromide 0.5 mg/ml and visualisation under UV light (UVP INC.) as above.

For analysis of the *agrBDC* region, restriction enzyme *MseI* was used. For the

coagulase gene *AluI* was used and for protein A gene *RsaI* was used.

2.9 TOXIN DETECTION

2.9.1 Molecular methods

2.9.1.1 Multiplex PCR for enterotoxins A-E, exfoliative toxins A-B and toxic shock syndrome toxin-1 (TSST-1)

This was based on two separate multiplex PCR for enterotoxins A-E, exfoliative toxins A-B and toxic shock syndrome toxin-1 (TSST-1) genes (Prof. C. Gemmell, Department Bacteriology, Glasgow Royal Infirmary, personal communication) but different reaction conditions and PCR protocol were used.

A multiplex PCR was developed in this study for these eight different target genes. The primers used were SEA-1 and SEA-2 for enterotoxin A, SEB-3 and SEB-4 for enterotoxin B, SEC-3 and SEC-4 for enterotoxin C, SED-3 and SED-4 for enterotoxin D, SEE-2 and SEE-3 for enterotoxin E, ETA-1 and ETA-2 for exofoliative toxin A, ETB-1 and ETB-2 for exfoliative toxin B and TST-3 and TST-6 for toxic shock syndrome toxin-1 (**Table 2.11**). The PCR Reaction Mix prepared with 45 µl of 1.1 X PCR master mix (3.0 mM $MgCl_2$) (AB gene, Surrey, UK) contained; 1.25 units *Taq* DNA polymerase, 75 mM Tris-HCl (pH 8.8 at 25 °C), 20 mM $(NH_4)_2$ SO_4, 3.0 mM $MgCl_2$, Tween 20, 0.01% (v/v), 0.2 mM of each dNTP. The concentration of primers were: for *sea*, *seb*, *sec*, *see* and *tst*, 10 pM; for *sed*, 20 pM; for *etb*, 30 pM; and for *eta*, 50 pM. Template DNA was 1 µl of 1 in 20 dilution. Positive controls included the template DNA of 6 different control strains HUM-G1 to G6 which were mixed together and then 1 µl of DNAs mixture was used. The PCR amplification was 30 cycles of: denaturation at 95 °C for 30s, annealing at 55 °C for 30s, and extension at 72 °C for 1 min, followed by a final cycle of 72 °C for 5 min. Fifteen microlitres of the PCR-amplification products were analysed by electrophoresis by using an

agarose 1.5% (w/v) gel as above.

2.9.1.2 Multiplex PCR for α, γ and δ-toxin genes

A multiplex PCR was developed in this study for these three genes. The primers used were HLA-1 and HLA-2 for α-toxin, HLG-1 and HLG-2 for γ-toxin and HLD-1 and HlD-2 for δ-toxin (**Table 2.11**). The PCR reaction mix was prepared with the use of Ready-To-Go PCR Beads (Amersham Pharmacia Biotech, UK). The multiplex PCR was carried out in a 25 µl reaction volume. Each reaction mixture contained 1.5 U of *Taq* DNA polymerase, 10 mM Tris-HCl, (pH 9.0 at room temperature), 50 mM KCl and 1.5 mM $MgCl_2$, and 200 µM of each dNTP. The concentration of primers was: for HLA and HLD, 100 pM; for HLG, 25 pM. Template DNA was 4 µl of 1 in 20 dilution in distilled water (double-processed tissue culture water). Amplification was done by predenaturation at 94 °C for 3 min and then 30 cycles of: denaturation at 94 °C for 1 min 30s, annealing at 55 °C for 2 min and extension at 72 °C for 3 min. A final extension at 72 °C for 5 min was used. Fifteen microlitres of PCR products were electrophoresed in an agarose 1.2% (w/v) gel as above.

2.9.1.3 PCR for β-toxin gene

The primers used were HLB-1 and HLB-2 (**Table 2.11**). The PCR reaction mixture contained 45 µl of master mix (AB gene, Surrey, UK), plus 1.0 mM of $MgCl_2$ (final concentration of $MgCl_2$ was 2.5 mM). The concentration of primers were 100 pM, and 2 µl of template DNA used. Amplification was done by predenaturation at 94 °C for 3 min and then 30 cycles of: denaturation at 94 °C for 1 min 30s, annealing at 55 °C for 2 min and extension at 72 °C for 3 min. A final extension at 72 °C for 5 min was used. Fifteen microlitres of PCR products were electrophoresed in an agarose 1.2% (w/v) gel as above.

2.9.2 Biological methods

2.9.2.1 Enterotoxins A-D

The detection of staphylococcal enterotoxins A, B, C and D was done with Staphylococcal enterotoxin reversed passive latex agglutination (SET-RPLA) TD900 kit (Oxoid) according to the manufacturer's instruction. Isolates were inoculated into Tryptone soya broth and incubated at 37 °C for 18-24 h. After growth, test samples (culture supernates) were prepared by centrifugation at 15,000 xg for 3 min and then filtration through a 0.22 μm filter (Millipore). This assay depends on the agglutination of latex beads coated with antibody specific for the toxins.

2.9.2.2 α and β-toxins

The organisms were inoculated on Blood Agar Base No. 2 (Oxoid) containing washed rabbit red blood cells 5% (v/v) and incubated overnight at 37 °C for detection of α-toxin.

For detection of β-toxin, the organisms were streaked onto Blood Agar Base No. 2, (Oxoid) containing washed sheep red blood cells 5% (v/v) and incubated overnight at 37 °C. Haemolytic activity of this toxin was enhanced by incubation below 10 °C after treatment at 37 °C, to detect the "hot-cold" haemolysin (Glenny and Stevens, 1935).

2.10 GROWTH OF *S. aureus* STRAINS AT HIGH TEMPERATURES

Twelve typical avian, 12 atypical avian and 11 human strains (**Table 2.12**) were cultured overnight at 37 °C on nutrient agar plates then one colony was inoculated into 10 ml of nutrient broth in a universal bottle. These cultures were incubated in a water bath with shaking at 150 rpm at 44-45 °C or 46-47 °C. The turbidity of the culture was recorded after overnight incubation.

Table 2.12 Growth of *S. aureus* strains at 44-45 °C or 46-47 °C

Type	Isolates	No. of isolates
Typical avian	24, 52, 53, 85, 99, 100, 105, 122, 123, 170, 329, 330	12
Atypical avian	82, 83, 86, 496, 525, 562, 199, 214, 217, 235, 31178, 32528	12
Human	CN4575, RN4220, 8530, HUM-G1, HUM-G2, HUM-G3, HUM-G4, HUM-G5, HUM-G6, HUM-11, HUM-BB	11

2.11 SEQUENCE ANALYSIS

2.11.1 Sequencing reactions

PCR products from the *agrBDC*, coagulase, 16S rRNA and cysteine protease genes from a variety of isolates, some avian and some of human origin (**Table 2.13**) were purified as above (Section 2.6.6) and reaction mixtures shown below were assembled in thin-walled 0.5 ml tubes and provided to the sequencing service at the Molecular Biology Support Unit (MBSU) at the University of Glasgow.

Terminator Ready Reaction Mix (Perkin-Elmer, UK)	8 µl
Double stranded DNA	250-500 ng
Primer (each PCR product)	3.2 pmol
Distilled water	variable
Final Reaction Volume	**20 µl**

Primers for each of these sequencing reactions were the same as those used for PCR detection of these genes (**Table 2.11**).

2.11.2 Running the sequencing gel

An Applied Biosystems 377 or 373 automated sequencing machine (Perkin Elmer, UK) was used for all sequencing reactions. The sequencing service at the Molecular Biology Support Unit (MBSU) at the University of Glasgow performed the gel run and results were posted electronically. Briefly, the dried pellet was resuspended by vortexing in 4 µl of loading solution (Perkin-Elmer) containing deionised formamide and 50 mM EDTA in the ratio 5:1. The samples were then heated to 90 °C for 2 min and placed on ice until ready to load.

Table 2.13 Sequencing of different genes in *S. aureus* isolates

Sequence	Isolates[a]	No. of isolates
16S rRNA	24, 85, 99, 34, 98, 82, 496, RN4220	8
Coagulase	24, 85, 99, 2B, 31174, PD27, 34, 38, 82, 83, 86, 562, 496, 217, 235, 525, RN4220	17
agrBDC	24, 85, 99, 2B, 31174, PD27, 34, 38, 104, 595, 98, 35, 82, 83, 86, 562, 496, 32528, 31178, 217, 235, 525, RN4220	23

[a]All isolates were avian except strain RN4220 which was from a human.

2.11.3 Analysis of sequence

2.11.3.1 ABI Prism ™Edit View

ABI Prism™ Edit View was used to check the parameters used for the gel run, to allow the signal strengths to be evaluated and also to allow the quality of the sequence data to be verified manually.

2.11.3.2 SeqEd

Contiguous sequences were generated using SeqEd V1.0.3. The ABI electropherograms were loaded into the program, filtered to remove any low quality data and then assembled to generate a consensus sequence. Finally any anomalies were rectified by evaluation of the trace data.

2.11.3.3 Genejockey II

Genejockey II sequence processor (Biosoft, Cambridge, UK) was used for a wide variety of purposes. For example, nucleotide sequences (not trace data) were aligned, translated or restriction sites identified. Peptide sequences were also analysed as appropriate. For example, peptide plots could be derived and amino acid sequences compared.

2.11.4 Internet tools

2.11.4.1 National Center for Biotechnology Information (NCBI)

Query sequences were routinely compared to those at the NCBI (http://www.ncbi.nlm.nih.gov/) using the Basic Local Alignment Tool (BLAST) search engines. Sequences of interest were downloaded and manipulated as Genejockey II files.

2.11.4.2 S. aureus genomic databases and tools

Query sequences were compared to the incomplete *S. aureus* genome database at:

(http://www.sanger.ac.uk/Projects/S_aureus/)
Contigs were searched using the Alpha-powered BLAST search engine using the TBLASTN blast executable at:
(http://www.sanger.ac.uk/Projects/S_aureus/blast_server.shtml).
Contigs of interest were downloaded and manipulated as Genejockey files.

The NCBI web site was also used to identify and further characterise microbial genomes. A list of genomes can be found at:
http://www.ncbi.nlm.nih.gov/Microb_blast/unfinishedgenome.html

3. RESULTS

3.1 IDENTITY OF ISOLATES

During the course of this study a total of 86 avian isolates of *S. aureus* were obtained at different times and from different sources. Some of these strains were provided as avian *S. aureus* and had already been well characterised (e.g. N. Ireland isolates from Dr. McCullagh). Others were less well characterised. In additional a number of other avian, human and animal coagulase-positive and coagulase-negative species were obtained, to serve as control organisms in the various test procedures. The identity of most strains, except some of the isolates already typed by PFGE by McCullagh *et al.* (1998), was determined or confirmed by cultural and biochemical tests.

3.1.1 Cultural and biochemical properties

On tryptic soy agar, nutrient agar, non-selective blood agar, brain heart infusion agar and mannitol salt agar, isolated colonies of all isolates were 1 to 3 mm in diameter within 24-48 h at 37 °C. The colonies of all strains were pigmented yellow, except for the avian *S. cohnii* isolate 3B and the avian *S. sciuri* isolates 31038, 31040 and 31041. These produced cream colonies. Microscopically, the bacteria were Gram-positive cocci forming "grape-like" clusters, non-motile and non-spore forming. The DNase test was positive for all coagulase-positive isolates. The catalase test was positive for all isolates.

3.1.2 Biotyping of N. Ireland strains

The biotyping results of the 40 avian *S. aureus* N. Ireland strains were kindly provided by Dr. J. J. McCullagh and are summarised in **Table 3.1**. Biotyping was done according to the schemes of Devrise (1984) and Isigidi *et al.* (1990). According to these results, 32 of the strains had the poultry biotype, seven strains had the non-host specific (NHS) biotype and one strain had the slaughterhouse biotype.

Table 3.1 Biotyping results for the avian *S. aureus* strains from N. Ireland (Dr J. J. McCullagh)

Biotype	Isolate	No. of isolates
Poultry	24, 35, 36, 38, 77, 82, 83, 85, 86, 98, 99, 100, 104, 105, 122, 123, 564, 572, 580, 582, 595, 596, 597, 598, 3, 20, 25, 30, 48, 51, 52, 53	32
[a]NHS	11, 34, 496, 31, 33, 47, 525	7
Slaughterhouse	562	1

[a]Non-host specific biotype.

3.1.3 Rapidec Staph

Rapidec Staph (bioMérieux) is designed for the identification of the main staphylococcal species isolated from human specimens. The results for the different avian isolates and other, control strains used in this study are summarised in **Table 3.2**. All of the avian isolates were confirmed as *S. aureus* except for isolate SV132, obtained from a Harris Hawk, which was *S. intermedius*. Also this system was not able to identify isolates 3B, 31037, 31038, 31040, 31041 and 31051 to species level. All avian, human and animal *S. aureus* strains (**Table 3.2**) gave an identical biochemical profile by Rapidec Staph included positive fluorescent reaction, positive reaction (yellow) for alkaline phosphatase (PAL) test, positive reaction for catalase test, negative reaction (yellow) for β-galactosidase (β GAL) test, except for these strains: 1) avian strain 31039 showed a negative reaction (colourless) for the alkaline phosphatase (PAL) test but the other tests were similar to other *S. aureus* isolates. 2) bovine strain BOV-32276 showed a red colour for β-galactosidase (β GAL) test, but according to the manufacturer's report for *S. aureus* strains reaction for this test is colourless (negative) and for *S. intermedius*, *S. xylosus* and *S. saprophyticus* it is pink or red.

3.1.4 API Staph

Identification was made by using the Analytic Profile Index with the API Staph (bioMérieux) system. The pattern of the reactions are obtained and coded into a 7-digit numerical profile. The results for the different avian isolates and other controls strains used in this study are summarised in **Table 3.3**. All of the avian isolates were confirmed as *S. aureus* except for: isolate SV132 obtained from a Harris Hawk which was *S. intermedius*; isolates 31037 and 3B that were obtained from chickens and were *S. cohnii*; isolates 31038, 31040 and 31041 that were obtained from chickens and were *S. sciuri*; and isolate 31051 that was obtained from chicken and was *S. caprae*. According to these results all avian, human and animal *S. aureus* strains (**Table 3.3**) gave analytic profile index 6736153, except for avian isolates 31036, 31039, E and 32528 which gave

Table 3.2 Results of Rapidec Staph for different isolates of staphylococci

Isolate	Species (No. of isolates)
2A, 2B, 3A, 4, 5, 6, 7A, 7B, 8, 31174, 31175, 31176, 31177, 31178, 31179, 31180, 31181, 31182, 31183, 31036, 31039, 32528, E, K, SV6, SV289, NFR, 199,214, 217, 235, PD486, PD458, PD380, PD27, 24, 34,35, 36, 38, 77, 82, 83, 85, 86, 98, 99, 100, 104, 105, 122, 123, 496, 562, 564, 572, 580, 582, 595, 596, 597, 598, 3, 11, 20, 25, 30, 48, 51, 52, 53, 88, 170, 172, 259, 329, 330, 352, 373, 390, 410, 451, 31, 33, 47, 525, HUM-2, HUM-3, CN4575, HUM-11, 8530, RN4220, HUM-108, HUM-109, HUM-G1, HUM-G2, HUM-G3, HUM-G4, HUM-G5, HUM-G6, HUM-271, HUM-275, HUM-282, HUM-284, HUM-294, HUM-298, HUM-BB, HOR-31912, BOV-32276, ZM-7, BOV-65, BOV-66, BOV-67	*S. aureus* (113)
SV132, DOG-32236, RAB-32253	*S. intermedius* (3)
134, 1-Tl, BOV-32298, BOV-32304	*S. epidermidis* (4)
132	*S. saprophyticus* (1)
31037, 31038, 31040, 31041, 31051, 3B, DUK-31915, BOV-32265	*Staphylococcus* spp. (8)

Table 3.3 Results of API Staph for different isolates of staphylococci

Isolate	Species (No. of isolates)
2A, 2B, 3A, 4, 5, 6, 7A, 7B, 8, 31174, 31175, 31176, 31177, 31178, 31179, 31180, 31181, 31182, 31183, 31036, 31039, 32528, E, K, SV6, SV289, NFR, 199, 214, 217, 235, PD486, PD458, PD380, PD27, 24, 34, 35, 36, 38, 77, 82, 83, 85, 86, 98, 99, 100, 104, 105, 122, 123, 496, 562, 564, 572, 580, 582, 595, 596, 597, 598, 3, 11, 20, 25, 30, 48, 51, 52, 53, 88, 170, 172, 259, 329, 330, 352, 373, 390, 410, 451, 31, 33, 47, 525, HUM-2, HUM-3, CN4575, HUM-11, 8530,RN4220, HUM-108, HUM-109, HUM-G1, HUM-G2, HUM-G3, HUM-G4, HUM-G5, HUM-G6, HUM-271, HUM-275, HUM-282, HUM-284, HUM-294, HUM-298, HUM-BB, HOR-31912, BOV-32276, ZM-7, BOV-65, BOV-66, BOV-67	*S. aureus* (113)
SV132, DOG-32236, RAB-32253	*S. intermedius* (3)
31037, 3B	*S. cohnii* (2)
31038, 31040, 31041	*S. sciuri* (3)
31051	*S. caprae* (1)
134, 1-Tl, BOV-32298, BOV-32304	*S. epidermidis* (4)
132	*S. saprophyticus* (1)
DUK-31915	*S. hominis* (1)
BOV-32265	*S. warneri* (1)

profiles of 6736113, 6736113, 6736173 and 6726153 respectively, and human isolates HUM-108 and HUM-109 which gave 6736111 and 6736143 respectively, and animal isolate HOR-31912 which gave 6336151. However, these numerical profiles still gave acceptable identification as *S. aureus*.

3.1.5 Identification of *S. aureus* by PCR

Nucleic acid amplification by PCR has been applied to the detection and identification of microbes and is becoming widely used for detection for diagnostic purposes in clinical research and diagnostic laboratories. Some PCR methods have been developed for rapid identification of *S. aureus* strains.

3.1.5.1 The S. aureus-specific PCR assay

The *S. aureus*-specific PCR assay has been developed for rapid identification of *S. aureus* isolates by Martineau *et al.* (1998). In the present study, oligonucleotide primers SA442-1 and SA442-2 (**Table 2.11**) as described by Martineau *et al.* (1998) were used. The PCR condition described by Martineau *et al.* (1998) were optimised for use with the avian, human or animal *S. aureus* strains.

All the avian, human and animal isolates identified as *S. aureus* in the present study gave a single similar amplimer of the expected size of 108 bp. Examples of the results obtained by this PCR method are shown in **Figure 3.1**. None of the avian, human or animal coagulase-negative or *S. intermedius* isolates showed any fragment by this method (e.g. lanes 11, 14, 15 in **Figure 3.1**).

3.1.5.2 PCR for nuclease region

The nuclease gene product is an extracellular thermostable endonuclease which degrades both DNA and RNA. *S. aureus*-specific nuclease primers were NUC1 and NUC2 (**Table 2.11**) and were expected to give an amplimer of size 0.3 kb as described previously by Brakstad *et al.* (1992). This study optimised and modified the PCR conditions described by Brakstad *et al.* (1992).

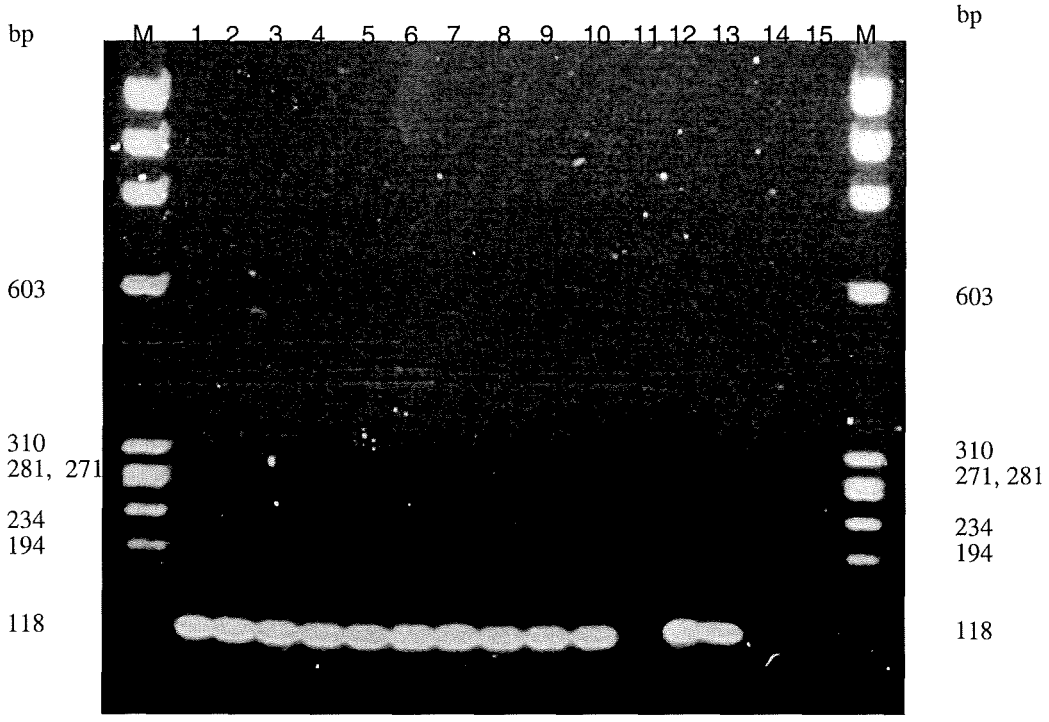


Figure 3.1 PCR for rapid identification of *S. aureus* isolates. Lanes M: ϕX174 RF DNA/Hae III Fragments DNA Ladder. Lanes 1-10: avian *S. aureus,* isolates 24, 34, 35, 36, 38, 77, 82, 83, 86 and 98, respectively. Lane 11: avian *S. cohnii*, isolate 31037. Lanes 12: human *S. aureus,* isolate RN4220. Lane 13: animal *S. aureus,* isolate HOR-31912. Lane 14: human *S. epidermidis,* isolate 134. Lane 15: animal *S. warneri,* isolate BOV-32265.

All the 86 avian, human and animal *S. aureus* isolates gave a single similar amplimer of size 0.3 kb (**Figure 3.2**). The results agreed with the biological method for nuclease detection in the DNase test except that a human isolate HUM-G5 did not give a positive PCR result but gave a positive result with the biological method. None of the avian and animal *S. intermedius* isolates SV132, DOG-32236 and RAB-32253 and the six avian and the three human coagulase-negative isolates or the animal *S. hominis* isolate DUK-31915 showed a nuclease gene amplimer (e.g. lane 35 in **Figure 3.2**).

3.2 PLASMIDS

Plasmid profile analysis is technically simple and represents the first DNA-based typing method applied to *S. aureus* (McGowan *et al.,* 1979; Locksley *et al.,* 1982). This study investigated the use of this method for discrimination between avian isolates of staphylococci.

3.2.1 Plasmid DNA extraction

The plasmid preparation was done with a QIA Prep Spin Plasmid Kit (QIAGE). This method is normally used for Gram-negative organisms but, in this study, was modified for staphylococci by including lysostaphin in the lysis step (See Section 2.4.1).

Seventy five avian *S. aureus* strains isolated from five different sources were examined for their plasmid content using the above method, followed by agarose gel electrophoresis to visualise the plasmid bands. Plasmids were detected in 95% of the strains and only isolates 31178, 32528, 199 and 214 did not have any plasmids. Examples of some profiles are shown in **Figure 3.3**. The size of the plasmid bands ranged from 1.6 kb to 16 kb. Seven different profiles were evident in the avian *S. aureus* isolates (**Table 3.4**). Five of the 75 avian *S. aureus* isolates (7%) contained a single plasmid. These isolates were 31039, 82, 496, 562 and 525. Twelve of the 75 avian *S. aureus* isolates (16%) contained two

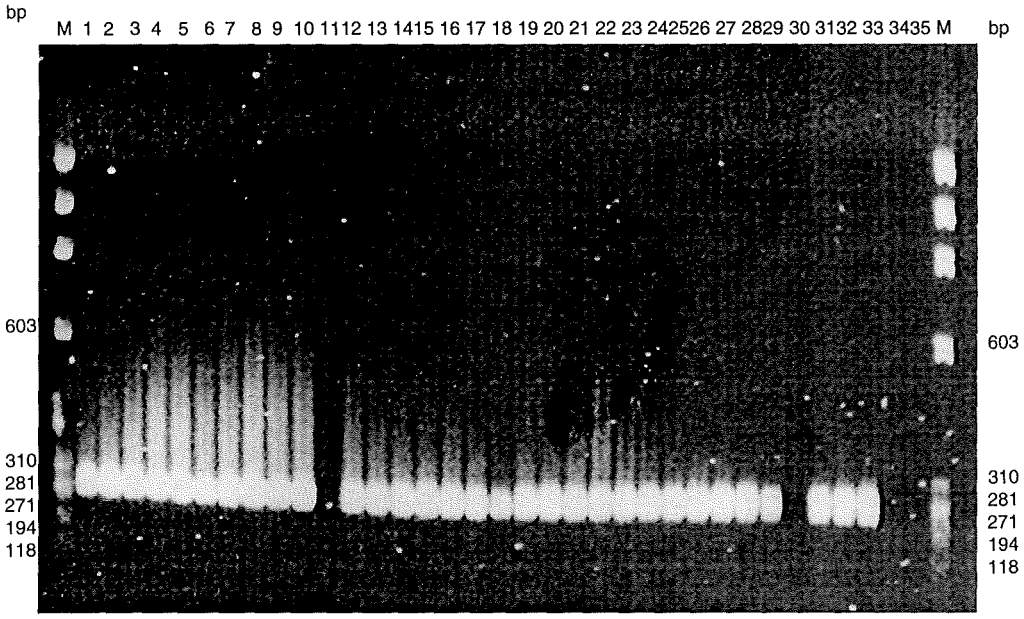


Figure 3.2 Examples of PCR amplification of nuclease gene fragment for *S. aureus* isolates. Lanes M: ϕX174 RF DNA/Hae III Fragments DNA Ladder. Lanes 1-10 and 12-29: avian *S. aureus* isolates 24, 34, 35, 36, 38, 77, 82, 83, 85, 86, 98, 99, 100, 104 105, 122, 123, 496, 562, 564, 572, 580, 582, 595, 596, 597, 2B, SV289 and SV6, respectively. Lane 11: avian *S. cohnii* isolate 31037. Lane 30: avian *S. sciuri* isolate 31040. Lanes 31-32: animal *S. aureus* isolates HOR-31912 and ZM-7, respectively. Lanes 33-34: human *S. aureus* isolates RN4220 and HUM-G5, respectively. Lane 35: animal *S. hominis* isolate DUK-31915.

plasmid bands but of different sizes: 1.6 kb, 1.8, 2.8 kb, 4.4 kb or 16 kb. Forty-nine of the 75 avian *S. aureus* isolates (65%) had three plasmid bands ranging in size from 1.6 kb, 1.8 kb, 2.2 kb, 3 kb, 4.4 kb and 16 kb. Five of the 75 avian *S. aureus* isolates (6%) had four plasmid bands and the sizes were 1.6 kb, 1.8 kb, 3.5 kb, 3 kb, 4.4 kb, 6 kb or 16 kb.

The results show that most of the avian N. Ireland *S. aureus* isolates related to PFGE type 24 and those closely related to type 24 according to McCullagh *et al.* (1998), and most of Scottish and Sri Lankan isolates gave three plasmid bands of 1.6 kb, 4.4 kb and 16 kb (**Table 3.4**).

Plasmids profiles from avian coagulase-negative and human and animals coagulase positive and negative staphylococci are shown in **Tables 3.5**, **3.6**, **3.7** and **3.8**. There were some plasmid bands of similar size in avian *S. aureus* isolates and avian coagulase-negative, human and animal coagulase-positive and negative isolates (**Tables 3.5**, **3.6**, **3.7** and **3.8**).

3.2.2 Transformation of plasmids by electroporation

This was done to confirm that a 4.4 kb plasmid common in avian *S. aureus* conferred tetracycline resistance (See Section 3.3). The method of Lee (1995) was used. A 4.4 kb from avian *S. aureus* isolate 7A was extracted by the QIA Prep Spin Plasmid Kit (QIAGEN) as described in section 2.4.1 and transferred to avian *S. aureus* isolate 6 and human *S. aureus* isolate RN4220 tetracycline-sensitive strains by electroporation. Selection for tetracycline resistance followed by re-extraction of the plasmid and agarose gel electrophoresis demonstrated the presence of the 4.4 kb plasmid. The plasmid profiles of avian *S. aureus* 6, before and after transformation, and of donor strain 7A are shown in **Figure 3.4**.

Table 3.4 Distribution of plasmids in avian *S. aureus* isolates

Plasmid bands (kb)	Isolate	No. of isolates
[a]----	31178, 32528, 199, 214	4
1.6	31039, 82	2
2.2	496, 525	2
16	562	1
1.6+4.4	PD458	1
1.8+16	217	1
1.6+16	3A, 6, 235, 35, 36, 77, 104, 580, 595	9
2.8+16	K	1
1.6+4.4+16	2A, 2B, 4, 5, 8, 31174, 31175, 31176 31176, 3177, 31179, 31180, 31181, 31182, 31183, 31036, PD486, PD380, 24, 34, 38, 85, 86, 99, 100, 105, 122, 123, 572, 582, 596, 3, 11, 20, 25, 30, 31, 33, 47, 48, 51, 52, 53, 170, 172	45
1.6+1.8+16	E	1
1.6+2.2+16	598	1
1.6+3+16	SV6, 597	2
1.6+3+4.4+16	7A, 7B, 564	3
1.6+1.8+3.5+16	98	1
1.6+1.8+6+16	83	1

[a] These isolates did not have plasmids.

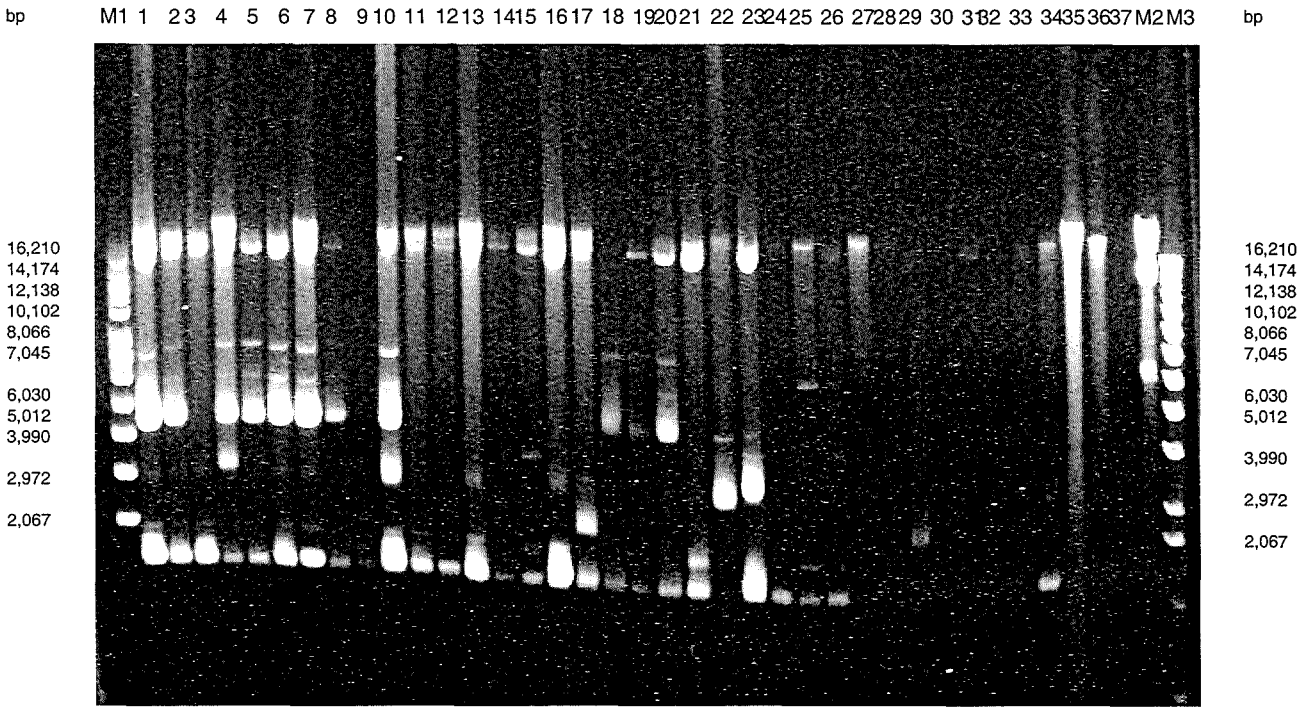


Figure 3.3 Examples of plasmid profiles of *S. aureus* isolates. Lane M1: Supercoiled DNA Ladder. Lanes 1-34: avian isolates, 2A, 4, 6, 7A, 31174, 11, 105, 31036, 31039, 564, 595, 104, 580, 35, 98, 597, 598, PD458, PD486, PD380, E, K, SV6, 82, 83, 86, 562, 496, 525, 32528, 31178, 199, 214 and 217, respectively. Lanes 35-36: human isolates, HUM-3 and HUM-G3. Lane 37: animal isolate HOR-31912. Lane M2: 5 Kb DNA Ladder. Lane M3: 1 Kb DNA Ladder. (see **Tables 3.4**, **3.6** and **3.7**)

Table 3.5 Distribution of plasmids in avian coagulase-negative staphylococci

Plasmid bands (kb)	Isolate	No. of isolates
*------	[a]31037, [b]31038, [b]31041	3
3	[b]31040	1
6.1	[c]31051	1
2.4+3	[a]3B	1

* These isolates did not have plasmids.
[a] *S. cohnii*, [b] *S. sciuri*, [c] *S. caprae*

Table 3.6 Distribution of plasmids in human *S. aureus* isolates

Plasmid bands (kb)	Isolate	No. of isolates
16	CN4575, 8530, HUM-11, HUM-G1, HUM-G2, HUM-G3, HUM-G4, HUM-3	8
*-----	RN4220	1

* This isolate did not have plasmids.

Table 3.7 Distribution of plasmids in animal coagulase-positive staphylococci

Plasmid bands (kb)	Isolate	No. of isolates
5+9.1	[a]BOV-32276	1
7+16	[b]RAB-32253	1
*-----	[a]HOR-31912, [a]BOV-65	2

[a]*S. aureus*, [b]*S. intermedius*
*These isolates did not have plasmid.

Table 3.8 Distribution of plasmids in animal coagulase-negative staphylococci

Plasmid bands (kb)	Isolate	No. of isolates
5	[a]BOV-32304, [b]BOV-32268	2
5+16	[a]BOV-32298	1
1.6+2.4+3.7+5+16	[c]BOV-32265	1

[a]*S. epidermidis*, [a]*S. chromogenes*, [c]*S. warneri*

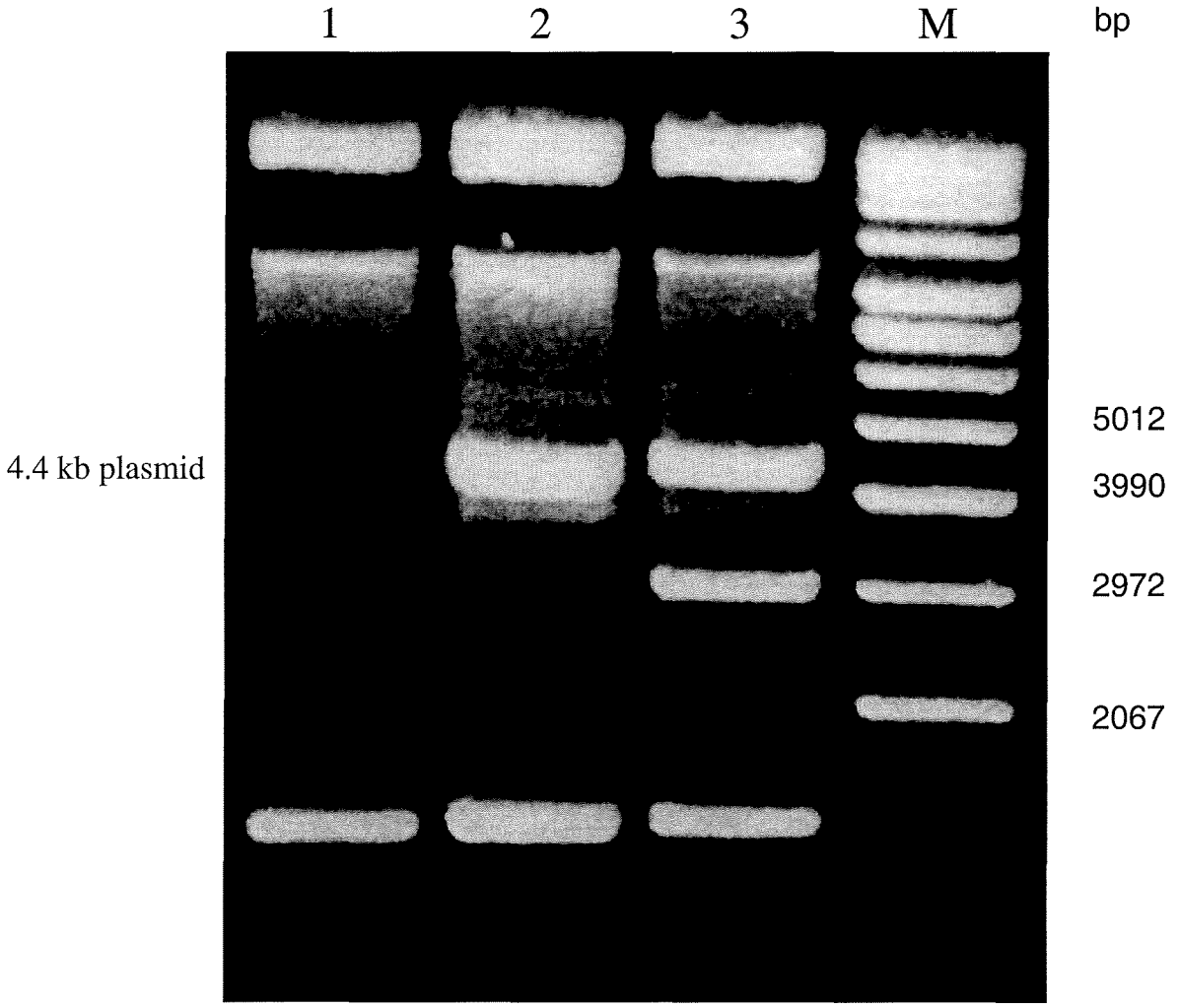


Figure 3.4 Plasmid profiles showing transformation of the 4.4 kb plasmid of *S. aureus* by electroporation. Lane 1: recipient avian isolate, 6 (before electroporation). Lane 2: recipient avian isolate, 6 (after electroporation). Lane 3: avian isolate, 7A (donor of 4.4 kb plasmid). Lane M: Supercoiled DNA Ladder.

3.3 ANTIBIOTIC SENSITIVITY PATTERNS

These were determined to see if they could be used for differentiation of avian *S. aureus* isolates and to find any relationship between antibiotic sensitivity patterns and plasmid profiles.

The antibiotic sensitivity results indicated that three of the 78 avian *S. aureus* isolates were sensitive to all the eighteen antibiotics tested by the ATB ANTIBIOGRAM (bioMérieux) method. These isolates were 31178, 31039 and K. Seventy-five (96%) of the 78 isolates were resistant to one or more of the antibiotics tested. Eleven (14%) of the 78 isolates were resistant to one antibiotic, either penicillin G, tetracycline or pefloxacin (**Table 3.9**). Ten (13%) of the isolates were resistant to two antibiotics that included tetracycline, pefloxacin, nitrofurantoin and erythromycin. Four (5%) of the isolates were resistant to three antibiotics namely kanamycin, tobramycin and gentamicin. Seventeen (22%) of the isolates were resistant to four antibiotics that included kanamycin, tobramycin, gentamicin, erythromycin, pefloxacin, lincomycin, tetracycline or penicillin G. Twenty-nine (37%) of the isolates were resistant to five antibiotics that included kanamycin, tobramycin, gentamicin, tetracycline and pefloxacin. Four (5%) of the 78 isolates were resistant to six antibiotics included kanamycin, tobramycin, gentamicin, pefloxacin, tetracycline and penicillin G (**Table 3.9**).

Of the 75 avian *S. aureus* isolates resistant to different antibiotics, fifty-three (71%) isolates were resistant to tetracycline, kanamycin, tobramycin and gentamicin, fifty (67%) isolates were resistant to pefloxacin, fourteen (19%) were resistant to penicillin G, two (3%) were resistant to erythromycin, one (1%) was resistant to nitrofurantoin and one (1%) isolate was resistant to lincomycin (**Table 3.9**).

The results showed that most of avian N. Ireland *S. aureus* isolates related to PFGE type 24 and closely related to type 24 according to McCullagh *et al.*

Table 3.9 Antibiotic resistance patterns of avian *S. aureus* isolates

Antibiotic resistance	Isolate	No. of isolates (%)
Tetracycline	7A, 7B, 31174, 31175, 31176, 31177, 31180, 31181	8 (10)
Pefloxacin	3A, 6	2 (3)
Penicillin G	32528	1 (1)
Tetracycline+Pefloxacin	2A, 2B, 4, 5, 31179, 31182, 31183, 31036	8 (10)
Tetracycline+Nitrofurantoin	8	1(1)
Erythromycin+Pefloxacin	SV6	1 (1)
Kanamycin+Tobramycin+ Gentamicin	82, 83, 496, 525	4 (5)
Kanamycin+Tobramycin+ Gentamicin+Pefloxacin	199, 214, 217, 235, 77	5 (6)
Kanamycin+Tobramycin+ Gentamicin+Tetracycline	582, 596	2 (3)
Kanamycin+Tobramycin+ Gentamicin+Penicillin G	35, 36, 98, 104, 562, 597, 580, 595, 598	9 (12)
Tetracycline+Erytromycin+ Lincomycin+Pefloxacin	E	1 (1)
Kanamycin+Tobramycin+ Gentamicin+Tetracycline+ Pefloxacin	PD486, PD458, PD380, PD27, 24, 34, 38, 85, 99, 105, 122, 123, 3, 11, 20, 25, 30, 31, 33, 47, 48, 51, 52, 53, 88, 170, 172, 410, 451	29 (37)
Kanamycin+Tobramycin+ Gentamicin+Tetracycline+ Pefloxacin+Penicillin G	86, 100, 564, 572	4 (5)

(1998), and most of Scottish and Sri Lankan isolates were resistant to tetracycline, kanamycin, tobramycin, gentamicin and pefloxacin (**Table 3.9**).

There was a strong correlation between the presence of a 4.4 kb plasmid as seen in the plasmid profile, and tetracycline resistance in avian *S. aureus* isolates (except for isolate E that did not apparently have a 4.4 kb plasmid but was resistant to tetracycline). This relationship was confirmed by plasmid transfer as described in section 3.2.2.

Antibiotic sensitivity patterns were also examined for the avian coagulase-negative, human *S. aureus* and animal coagulase-positive and coagulase-negative isolates. Of the six avian coagulase-negative strains, isolates 31037 and 31051 were sensitive to all 18 antibiotics but isolate 31038 was resistant to fusidic acid. Isolate 31040 was resistant to pefloxacin, isolate 31041 was resistant to lincomycin, and isolate 3B was resistant to fusidic acid and lincomycin. Of the seven human *S. aureus* isolates, CN4575, 8530, RN4220 were sensitive to all 18 antibiotics, but isolates HUM-2, HUM-3 and HUM-11 were resistant to penicillin G, isolate HUM-108 was resistant to penicillin G and erythromycin and pefloxacin. Of the five animal coagulase-positive isolates, HOR-31912 and ZM-7 were sensitive to all 18 antibiotics but isolate BOV-32276 was resistant to penicillin G, isolate DOG-32236 was resistant to penicillin G and tetracycline, and isolate RAB-32253 was resistant to penicillin G, lincomycin, pefloxacin and fusidic acid. Of the five animal coagulase-negative isolates, BOV-32268 and BOV-32304 were sensitive to all 18 antibiotics but isolate DUK-31915 was resistant to tetracycline, isolate BOV-32265 was resistant to fosfomycin and isolate BOV-32298 was resistant to penicillin G.

3.4 EXOPROTEINS PROFILES

Staphylococci produce a variety of different extracellular proteins acting as

either toxins, non-toxic enzymes, or enzyme activators. In this part of the project, extracellular proteins of avian *S. aureus* isolates were investigated to determine their usefulness in strain differentiation.

3.4.1 SDS-PAGE gels

After concentration of staphylococcal culture supernates, the exoprotein variation between sixty-seven avian *S. aureus* isolates was examined. For comparison, an avian *S. intermedius* isolate, three avian coagulase-negative isolates, six human *S. aureus* isolates and two human *S. epidermidis* isolates were included. Examples of exoprotein profiles are shown in **Figure 3.5**. Whereas the human strains (e.g. lanes 11 and 12, **Figure 3.5**) generally produced complex exoprotein profile, the profiles of the avian strains contained few bands. Also, the avian *S. intermedius* strain and the human *S. epidermidis* produced complex exoprotein profiles (lanes 3 and 4, **Figure 3.6**). A notable feature of the exoprotein profiles was that a single dominant protein band of 22 kDa was common to the majority of avian *S. aureus* strains. Only seven avian *S. aureus* isolates, 31177, 31178 (lane 5, **Figure 3.5**), 31039, 32528, K, 82 (lane 3, **Figure 3.5**), and 496 appeared to lack this band (**Table 3.10**). None of the three avian coagulase-negative, six human *S. aureus* (e.g. lanes 11 and 12, **Figure 3.5**, lanes 5 and 6, **Figure 3.6**) avian *S. intermedius* isolate, or the two human *S. epidermidis* isolates (lanes 3 and 4, **Figure 3.6**) examined by SDS-PAGE showed a band at the 22 kD position (**Table 3.10**).

3.4.2 Determination of N-terminal amino acid sequence of 22 kDa band

In view of the finding of a single major exoprotein produced by the majority of *S. aureus* avian strains, this protein, a putative virulence factor, was further investigated.

Concentrated culture supernates of avian strain 31179 was subjected to SDS-PAGE as above and bands were transferred onto a PVDF membrane (Bio-Rad) by the method of Matsudaira (1987). After brief staining with Coomassie

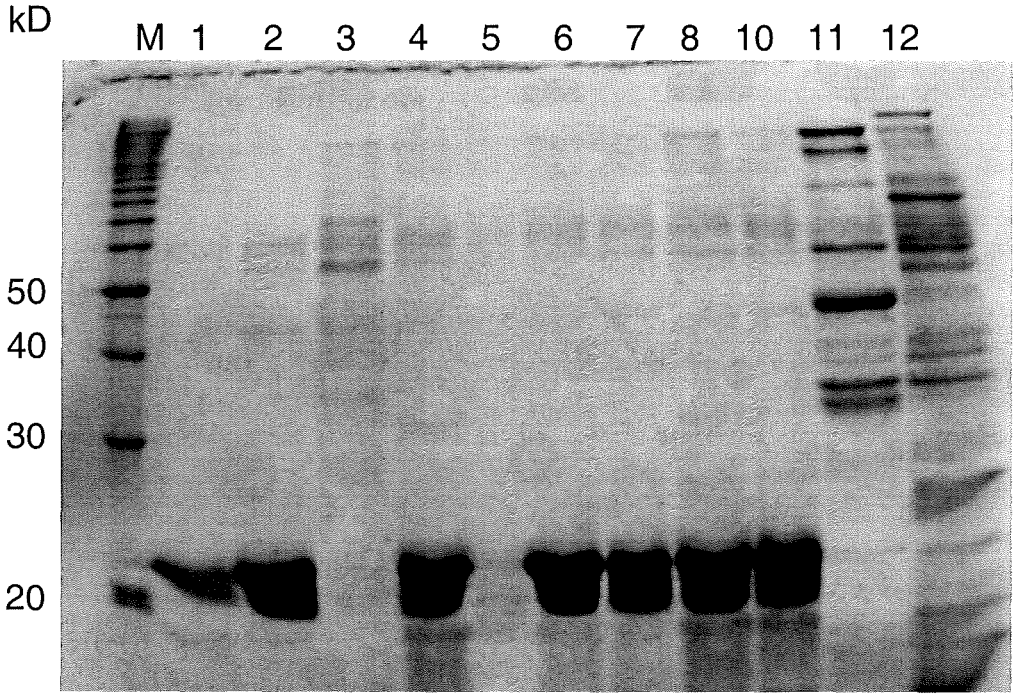


Figure 3.5 SDS-PAGE gel for exoproteins in *S. aureus* isolates. Lane M: 10 kDa Protein Ladder. Lanes 1-10 avian isolates 2B, 24, 82, 31174, 31178, 31179, E, 85, 35 and 86 respectively. Lane 11-12: human isolates RN4220 and 8325 (see **Table 3.10**).

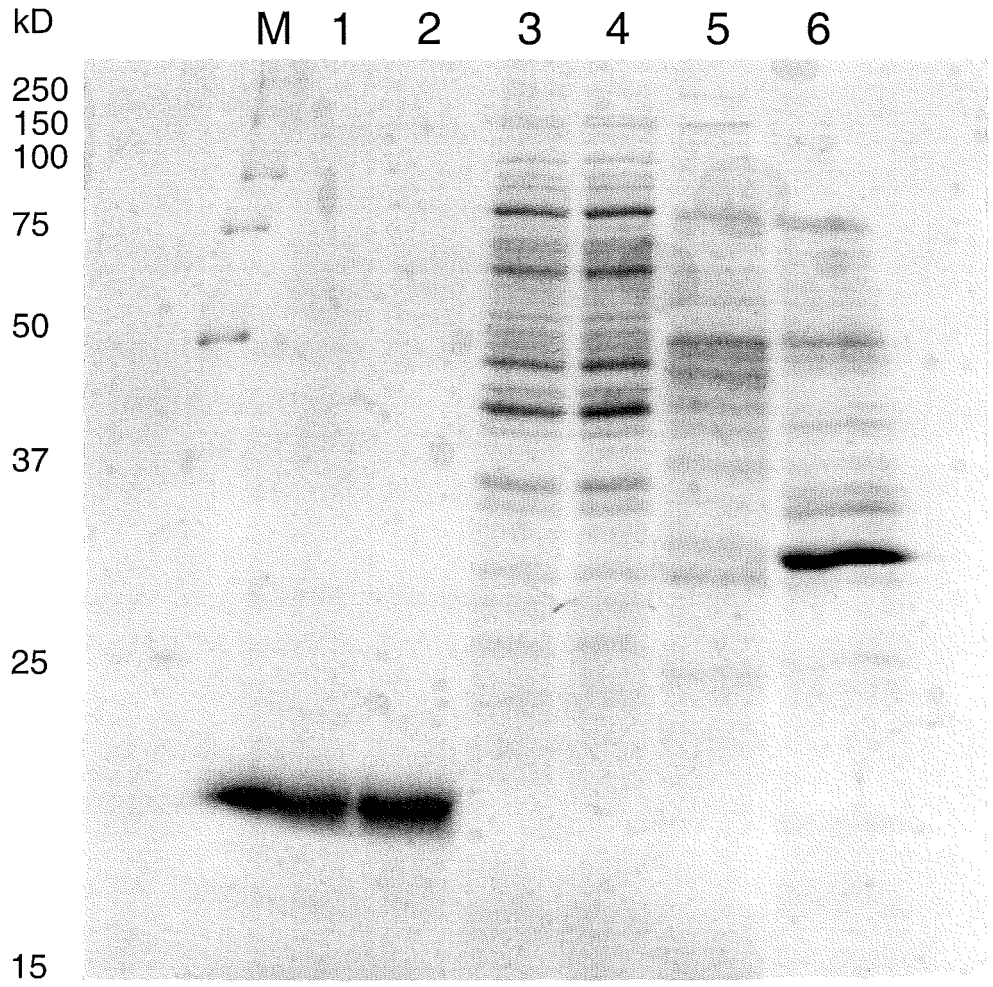


Figure 3.6 SDS-PAGE gel for exoproteins in *S. aureus* and *S. epidermidis* isolates. Lane M: Unstained Protein Ladder (Bio-Rad Laboratories UK). Lanes 1-2: avian *S. aureus* isolates 24 and 31179, respectively. Lanes 3-4: human *S. epidermidis* isolates 134 and 1-TL, repectively. Lanes 5-6: human *S. aureus* isolates 8530 and RN4220, respectively (see **Table 3.10**).

Table 3.10 Presence of 22kDa protein band in avian and human staphylococcal isolates as determined by SDS-PAGE

22kDa protein band	Isolates	No. of isolates
Positive (avian *S. aureus*)	2A, 2B, 3A, 4, 5, 6, 7A, 7B, 8, 31174, 31175, 31176, 31179, 31180, 31181, 31182, 31183, 31036, E, SV6, SV289, 24, 34, 35, 36, 38, 77, 83, 85, 86, 98, 99, 100, 104, 105, 122, 123, 562, 564, 572, 580, 582, 595, 596, 597, 598, 3, 11, 20, 25, 48, 51, 52, 53, 170, 172, 330, 390, 410, 451	60
Negative (avian *S. aureus*)	31177, 31178, K, 31039, 32528, 82, 496	7
Negative (avian *S. intermedius*)	SV132	1
Negative (avian coagulase-negative)	[a]31037, [b]31038, [b]31041	3
Negative (human *S. aureus*)	HUM-2, HUM-3, CN4575, HUM-11, 8530, RN4220	6
Negative (human *S. epidermidis*)	134, 1-TL	2

[a]*S. cohnii*, [b]*S. sciuri*

brilliant blue R-250, the area of the membrane containing the band of interest (22 kDa band) was cut out and sent for N-terminal sequencing at the Protein and Nucleic Acid Chemistry Laboratory (PNACL) at the University of Leicester. The N-terminal amino acids sequence of the 22 kDa band was found to be VRAQYVNQLKN. At the time this information was obtained (September 1999) no match was found in the protein databases (NCBI). However, in a paper by Takeuchi *et al.* (1999) published in June of that year, a protease was purified from the culture supernates of an avian strain of *S. aureus*. The protease had been characterised as a thiol (cysteine) protease and the N-terminal amino acid sequence was given as RAQYVNQLKNFKIRETQ. From these results, it appeared likely that the 22 kD band in culture supernates from avian *S. aureus* isolates represented an extracellular cysteine protease. This possibility was investigated further.

3.4.3 Screening for protease activity

3.4.3.1 Skim milk agar plates

Protease production by the 86 avian *S. aureus* isolates was examined by culture on skim milk agar plates. A clear zone was formed around the colonies of 80 of the isolates on skim milk agar plates. When protease-producing ability of these isolates was classified from + to +++ by the size of the clear zone, most of the isolates were judged to be +++. There was no clear zone around six avian *S. aureus* isolates, 31178, 31039, K, 32528, 82 and 496 or an avian isolate of *S. intermedius*. Most of these strains are the ones that lack a 22 kD band. The result therefore agreed with the presence of a 22 kD protease band by SDS-PAGE, except for isolate 31177, that was negative by SDS-PAGE gel but was positive on skim milk agar. Of the nine human *S. aureus* and four of the avian coagulase-negative isolates examined by skim milk agar plate culture, all of them were negative.

To determine whether the protease activity seen on skim milk was due to a cysteine protease or to some other type of protease, a specific cysteine protease inhibitor was used. Bacterial strains were streaked across skim milk agar plates and filter paper strips soaked in the specific cysteine protease inhibitor E64 at a concentration of 28 μM was laid across the streaks. After incubation, it was found that the specific cysteine protease inhibitor E64 inhibited the zone of clearing (protease activity) near to the filter paper strip where the inhibitor had diffused out into the medium (**Figure 3.7**). From these results, it appears the protease activity of the avian *S. aureus* strains seem on skim milk agar was due to a cysteine protease.

3.4.3.2 Effect of different reagents on protease activity

In further experiments to characterise the protease activity in avian strains different protease inhibitors (Roche Molecular Biochemicals, Germany) at recommended concentrations were investigated for their effect on the activity of culture supernates of avian *S. aureus* isolate 31179 that had a prominent 22 kD band. The protease activity was measured in a dye-release assay with azocasein as the substrate. Results are shown in **Table 3.11**. According to these results, the metalloprotase inhibitor (EDTA-Na_2) did not inhibit or stimulate the activity of protease. Protease activity was however, stimulated by the addition of L-cysteine. Cysteine protease inhibitor E64 (N-(N-(L-3-trans-carboxirane-2-carbonyl)-L-leucyl)-agmatine) almost completely inhibited the activity of the protease, whereas other protease inhibitors including pepstatin (aspartic acid protease inhibitor), APMSF [(4-amidino-phenyl) methane-sulfonyl fluoride] (serine protease inhibitor) and bestatin (amino peptidase inhibitor) had only minor inhibitory effects. These results confirm that the protease activity in the culture supernates of the avian strain is mainly due to a cysteine protease and that this protease is probably identical to the 22 kDa protein band seen in the culture supernates.

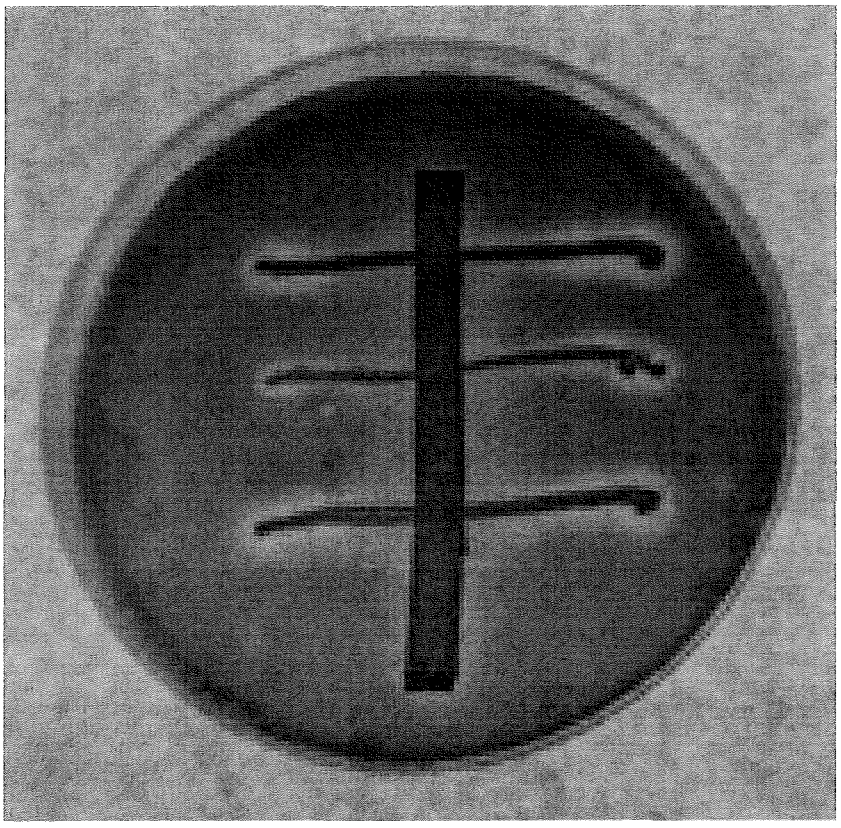


Figure 3.7 Demonstration of inhibition of protease activity of avian *S. aureus* isolates (from top to bottom, strains 2B, 24 and 85) by the cysteine protease inhibitor E64. The filter paper strip in the middle contained the cysteine protease inhibitor E64.

Table 3.11 Effect of specific inhibitors on the activity of the protease of *S. aureus* strain 31179 measured in dye release assay with azocasein as substrate

Reagent	Concentration	Effect (%)
None	-----	100
L-cysteine	50 mM	137.1
EDTA	1.3 mM	100
Pepstatin	1 μM	88.6
APMSF	20 μM	82.8
Bestatin	130 μM	77.1
E64	28 μM	8.5

3.4.3.3 Amplification of N-terminal sequence of cysteine protease

After determination of the N-terminal amino acid sequence of the 22 kD exoprotein band, an attempt was made to clone and sequence the gene encoding the protease. Primers PRS-1 and PRS-2 (**Table 2.11**) were designed from a combination of both N-terminal amino acids sequences (this study and Takeuchi *et al.,* 1999) to amplify an expected 54 bp fragment of the N-terminal amino acid sequence. A number of avian and human *S. aureus* isolates and human *S. epidermidis* isolates that were positive and negative for 22 kD band (**Table 3.10**) were selected for this test. These included avian *S. aureus* isolates 31179, 24 and 85 (which produce the 22 kDa band) and avian *S. aureus* isolate 82, human *S. aureus* isolates 8530 and RN4220 and human *S. epidermidis* isolate 134 (all of which were negative for 22 kDa band).

With avian *S. aureus* isolates 31179, 24 and 85, shown by SDS-PAGE to produce the 22 kD exoprotein, a 54 bp amplicon was obtained. Examples of this result are shown in **Figure 3.8**. The other avian and human *S. aureus* and *S. epidermidis* strains that lacked the 22 kD exoprotein were negative by PCR (e.g. lanes 3 and 4 **Figure 3.8**). In all strains amplified by this method, there were some other non-specific bands of different sizes (data not shown).

3.4.3.4 Southern blot analysis of the S. aureus genome

Next, the intention was to use the digoxigenin-labelled 54 bp fragment as a Southern blot probe (section 3.4.3.3) to detect the protease gene in the genome of avian *S. aureus* and then to clone the protease gene in a suitable vector.

Using the method described in Chapter 2, Section 2.5.8, the 54 bp probe produced from avian *S. aureus* isolate 31179 was hybridised to the genomes of avian *S. aureus* isolate 31179 and human *S. aureus* isolate RN4220. The latter was used as a control as it did not produce the 22 kDa exoprotein (**Table 3.10**). **Figure 3.9** shows the hybridisation results when the chromosomes were cut with *HindIII*, *XbaI* and *BamHI*. There were two bands, of about 1 and 2.3 kb when

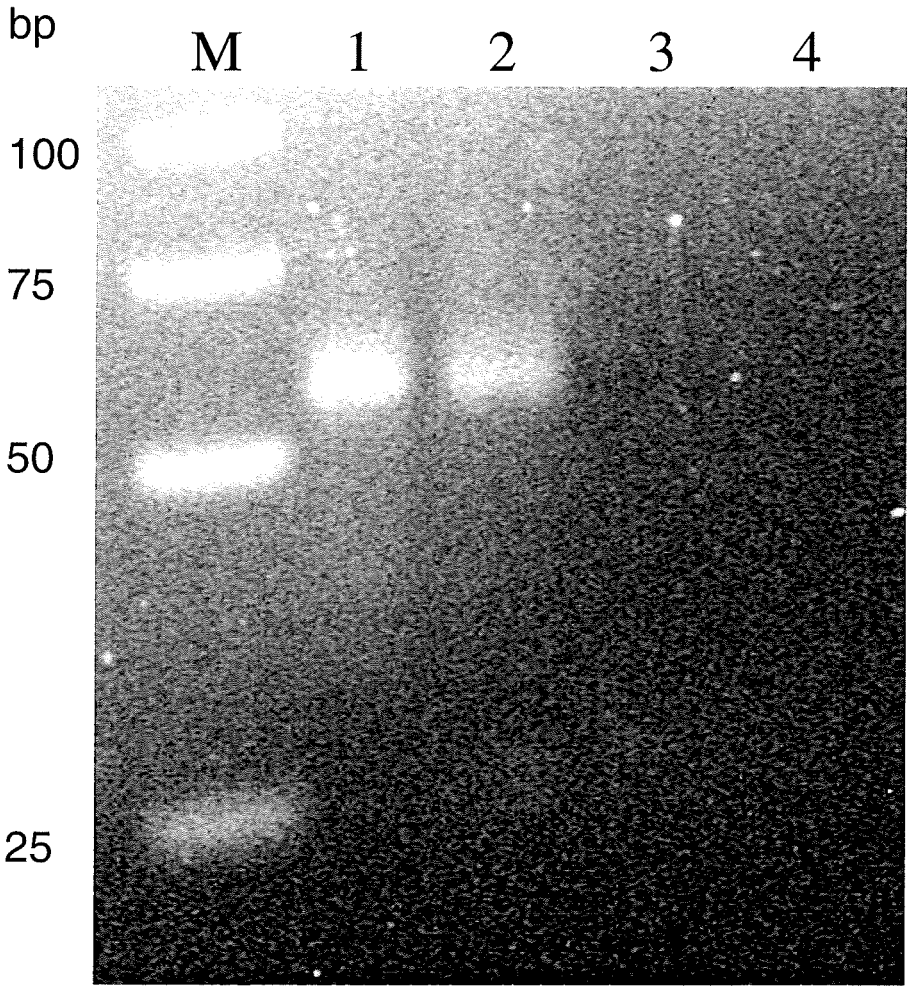


Figure 3.8 PCR of the N-terminal sequence of the 22 kDa protease for *S. aureus* and *S. epidermidis* isolates. Lane M: 25 bp DNA Ladder. Lane 1-2: avian *S. aureus* isolates, 31179 and 24. Lane 3: human *S. epidermidis* isolate, 134. Lane 4: human *S. aureus* isolate, RN4220.

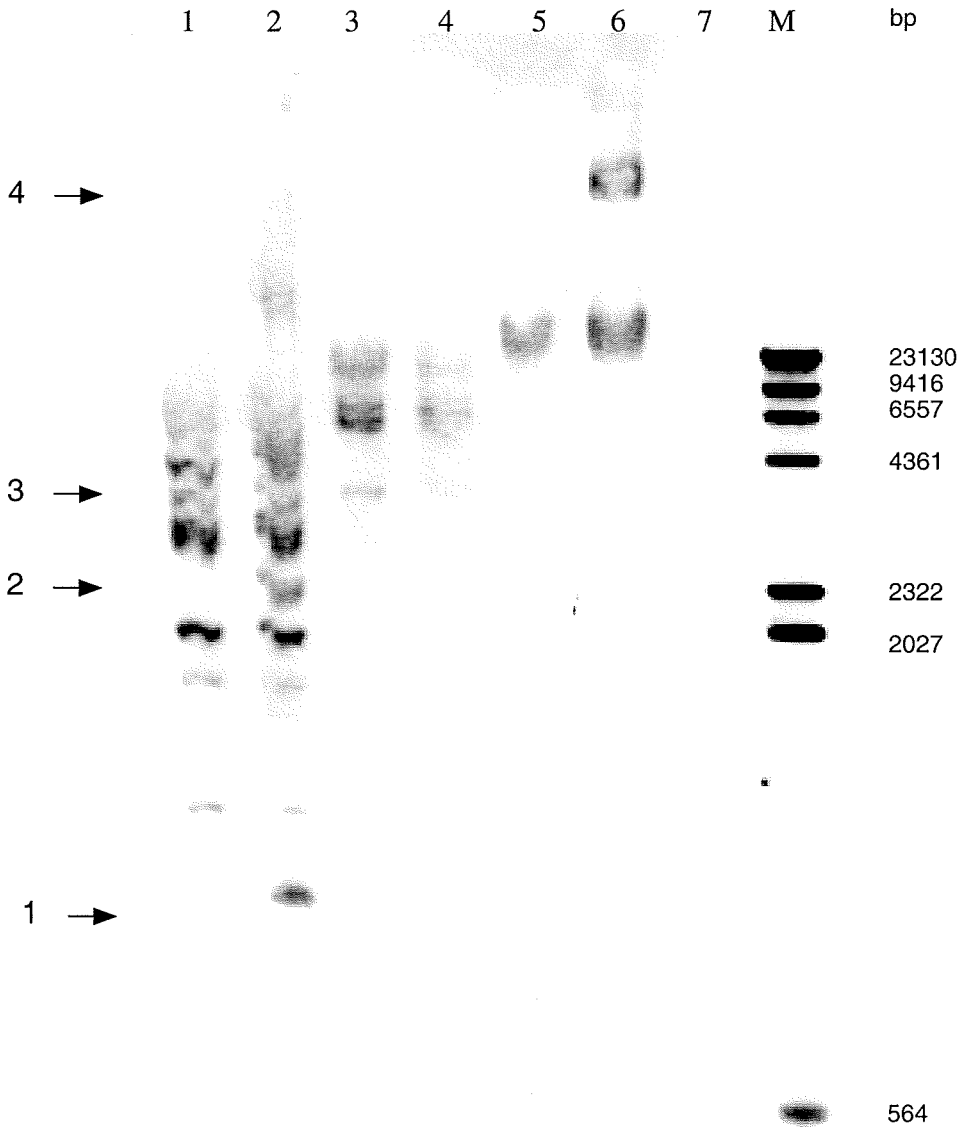


Figure 3.9 Hybridisation of a 54 bp probe derived from the 22 kDa protease of avian *S. aureus* isolate 31179 against chromosomal DNA of *S. aureus*. Lanes 1, 3, 5: human *S. aureus* isolate, RN4220 restricted by *HindIII*, *XbaI* and *BamHI* repectively. Lanes 2, 4 and 6: avian *S. aureus* isolate, 31179 restricted by *HindIII*, *XbaI* and *BamHI* respectively. Lane 7: *S. aureus* isolate, 31179 (labelling probe) (54bp N-terminal band) (the band ran out of the gel). Lane M: DNA Molecular-Weight Marker II DIG-labeled.

genomic DNA from avian *S. aureus* isolate 31179 was cut with *HindIII*, but these were not present in the human isolate (arrows 1 and 2 in **Figure 3.9**). There was also one extra band of about 4 kb when human isolate RN4220 was cut with *XbaI* (arrow 3 in **Figure 3.9**) and there was one extra band of high mol. wt in the avian isolate when cut with *BamHI* (arrow 4 in **Figure 3.9**). The labelling probe (54 N-terminal band) (lane 7 in **Figure 3.9**) did not show a band because its size was too small. These results indicated that it might be feasible to clone a 2.3 kb *HindIII* DNA fragment which might incorporate all or part of the protease gene. However, a different strategy was decided in the light of recent information, because the 54 bp of the N-terminal sequence was matched with a sequence identified as part of the *ecp* (extracellular cysteine proteinase) gene of human *S. epidermidis* strain 6746 (GenBank accession number AJ298299).

3.4.3.5 Amplification of cysteine protease gene

As stated above, the 54 bp N-terminal sequence of 22 kD exoprotein band was found to be 72% (39/54 bp) identical with regions of the *ecp* (extracellular cysteine proteinase) gene of *S. epidermidis* isolate 6746 (GenBank accession number AJ298299). From this information, primers SACP-1 and SACP-2 were designed (**Table 2.11**) to amplify a 522 bp region of the cysteine protease gene.

With this PCR method, avian *S. aureus* isolates 31179 and 24 and human *S. epidermidis* strain 134 gave a single similar amplicon of size ~500 bp (**Figure 3.10**). All these products were sequenced by Molecular Biology Support Unit (MBSU) at the University of Glasgow (see section 2.10.1). Human *S. aureus* isolates RN4220 and 8530 did not show bands of this size with the above primers.

3.4.3.6 Sequence analysis of cysteine protease gene

Regions of cysteine protease consensus sequences of the 522 bp sequence obtained for avian *S. aureus* isolates 24 and 31179 and the 522 bp sequence obtained for the human *S. epidermidis* isolate 134 were compared to other

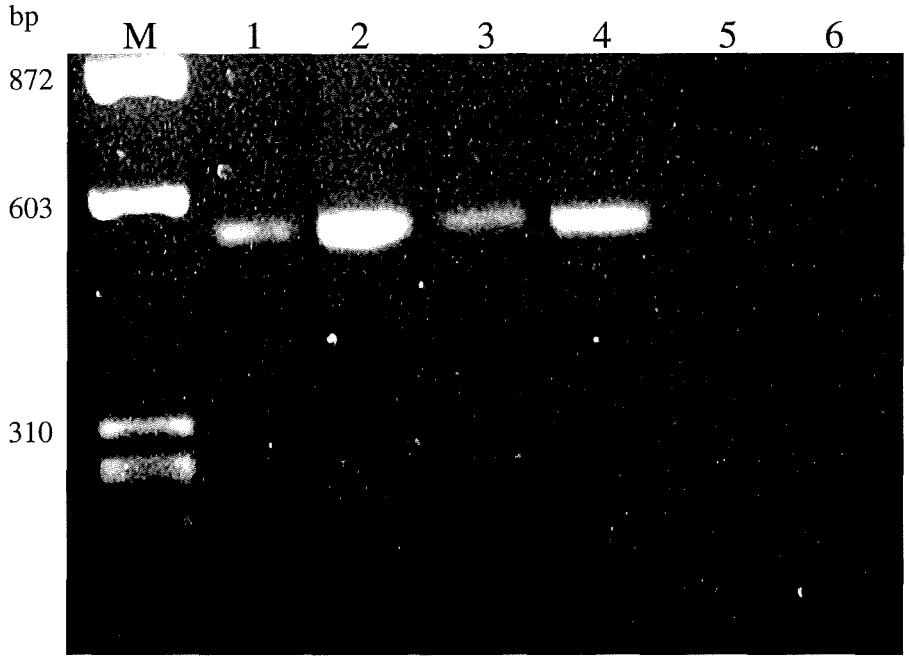


Figure 3.10 PCR amplification of extracellular cysteine protease gene. Lane M: ϕX174 RF DNA/*Hae III* Fragments DNA Ladder. Lane 1: human *S. epidermidis* isolate 134. Lanes 2-4: avian *S. aureus* isolates 31179, 24 and 85, respectively. Lanes 5-6: human *S. aureus* isolates 8530 and RN4220, respectively.

sequences in the GenBank using the BLASTn search engine. The sequences for strains 24 and 31179 were 100 % identical (**Appendix III-a**) and showed 80% (420/522 bp) identity with the published sequence for the *S. epidermidis ecp* gene of isolate 6746 (GenBank accession number AJ298299), 42% (221/522 bp) identity with the *scp* cysteine protease (staphopain) gene of human *S. aureus* subsp. *aureus* isolate N315 (GenBank accession number AP003135). Similarly, there was 42% (221/522 bp) identity with the *scp* cysteine protease (staphopain) gene of human *S. aureus* subsp. *aureus* strain Mu50 (GenBank accession number AP003363). The corresponding sequence of the *ecp* gene of human *S. epidermidis* isolate 134 used in the present study showed 100% identity with the published sequence for *S. epidermidis ecp* gene of isolate 6746.

3.4.3.7 Gelatin gels

In section 3.4.1, it was reported that a 22 kDa band representing an extracellular cysteine protease was found in the culture supernates of the majority of avian *S. aureus* strains. An investigation was therefore undertaken to determine whether differences in extracellular proteases could be used as an aid to strain discrimination. For this investigation, strains belonging to PFGE type 24 (McCullagh *et al.,* 1998) were chosen. This is the commonest PFGE type and, to date, there is no known way to discriminate between these strains. When protease activity in culture supernates was examined by the gelatin gel method, most of the isolates examined showed similar protease profiles. Examples of such profiles are shown in **Figure 3.11**. Although the manner in which the protease separated on the gel was not ideal, some differences were evident between avian *S. aureus* strains of PFGE type 24. For example, isolates 85, 123, 572, 582 and 31174 had slightly different patterns from those of the other isolates examined by this method (lanes 1, 4, 5, 6 and 9, **Figure 3.11**). These thirteen isolates examined here were identical by other biological and molecular typing methods. This procedure therefore seemed to offer the possibility of some discrimination between type 24 isolates, but this would need to be established by repeat analysis.

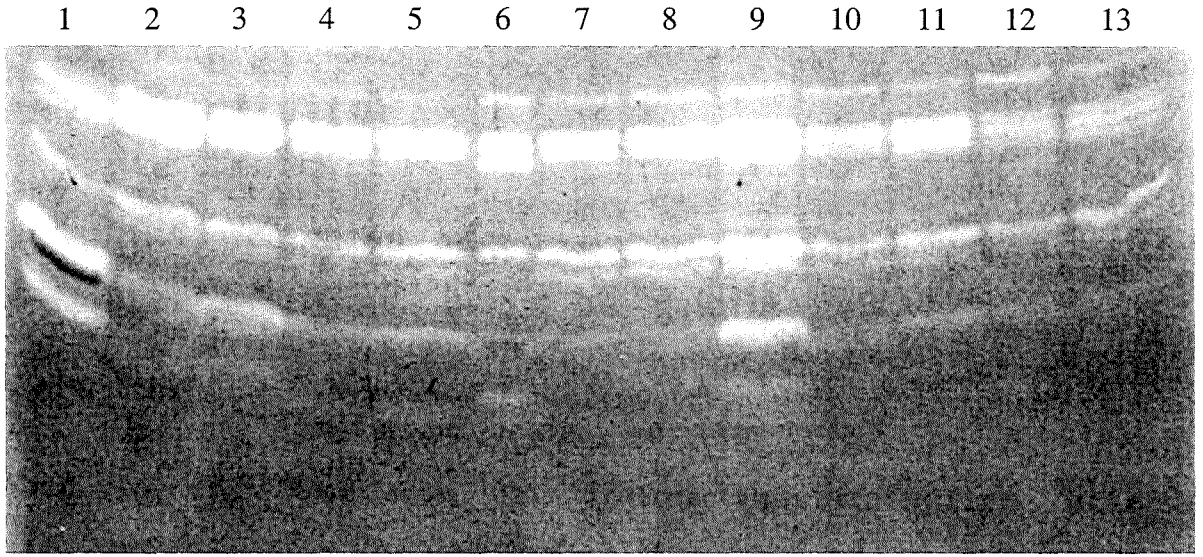


Figure 3.11 Gelatin gel for protease activity in culture supernates of avian *S. aureus* isolates of PFGE type 24. Lanes 1-13: isolates 85, 105, 122, 123, 572, 582, 596, 4, 31174, 31175, 31176, 31179 and 31180, respectively.

3.5 FINGERPRINTING OF ISOLATES BY PCR METHODS

One of the main aims of this project was to develop rapid and discriminating methods for the characterisation of avian isolates of *S. aureus* and to determine how these isolates differ from *S. aureus* strains from other sources. A number of PCR fingerprinting methods were investigated.

Some strains were typed by these methods on several occasions with essentially the same results, showing that the methods were highly reproducible. PCR products from a selection of the following strains e.g. avian *S. aureus* strains 24, 34, 11, 77, 85, 82, 83, 86, 496, 562, 595, 2A, 2B, 31174, 31177, 31181, human isolates 8530 and RN4220 or animal isolate BOV-65 were included in all gels, as "in-house" standards to facilitate the comparison between strains and for the identification of particular types of profile.

3.5.1 PCR-ribotyping

PCR-ribotyping method 1 involved the use of universal primers GIRRN and LIRRN (**Table 2.11**) that were designed to amplify the spacer region between the 16S and 23S rRNA genetic loci as described by Jensen *et al.* (1993). This study modified and optimised the PCR conditions described by Appuhamy *et al.* (1997) for use with *Haemophilus somnus* isolates (section 2.6.9.1).

All 86 avian *S. aureus* isolates gave fingerprints with approximately nine major bands ranging from ~0.4-~0.8 kb. Examples are shown in **Figure 3.12.** On the basis of six distinct bands, twelve groups were recognised for the 86 avian *S. aureus* isolates and each was assigned a number (**Table 3.12**). Group **1** included the majority, 63 (73%) of the isolates. When the N. Ireland isolates were examined, the results showed that all isolates designated as type 24 and closely-related to type 24 (according to McCullagh *et al.,* 1998) were in group **1**. Also, most of the Scottish isolates except isolates 31178, 31039 and 32528 and most of the English isolates except isolates K and NFR and all of the Sri Lankan isolates were in this group (**Table 3.12**). Group **2**, contained eight (9%) isolates;

of the N. Ireland isolates, all isolates designated as possibly-related to PFGE type 24 and some of the isolates designated as unrelated to type 24 (according to McCullagh *et al.,* 1998) were in this group. Group **3** contained four isolates (5%). All isolates in this group were N. Ireland strains designated as unrelated to PFGE type 24 (according to McCullagh *et al.,* 1998). Groups **4** and **5** contained two (2%) isolates each. Two of the American isolates were in group **4** and two N. Ireland isolates unrelated to PFGE type 24 (according to McCullagh *et al.,* 1998) were in group **5**. Groups **6, 7, 8, 9, 10, 11** and **12** each contained one (1%) isolate, from different sources (**Table 3.12**).

The amplified products of eight human *S. aureus* isolates (e g. lanes 35 and 36 in **Figure 3.12**) showed four different patterns which were distinct from those of avian *S. aureus* isolates, except for isolates HUM-2 and HUM-3. These strains gave patterns that were similar to group **1** of the avian *S. aureus* isolates (**Table 3.12**). Avian coagulase-negative isolates (e.g. lane 38 in **Figure 3.12**), animal coagulase-positive and negative and human coagulase-negative (e.g. lane 37 in **Figure 3.12**), also showed distinct patterns (**Table 3.12**).

Results obtained with PCR-ribotyping method 1 for the chicken isolates of *S. aureus* from N. Ireland and the other, atypical avian strains not from N. Ireland are included in summary **Tables 3.20** and **3.21**, respectively.

PCR-ribotyping method 2 was done with species-specific primers STAA-AVI and STAA-AVII (**Table 2.11**) as designed by Forsman *et al.* (1997) for use with bovine mastitis *S. aureus* strains. The primers are complementary to conserved sequences near the 3′ end of the 16S and the 5′ end of the 23S rRNA genes, respectively, and were used to amplify the 16S-23S intergenic spacer regions for bovine mastitis pathogens (Forsman *et al.,* 1997). This study modified and optimised the PCR conditions described by Forsman *et al.* (1997) (section 2.6.9.1).

Table 3.12 Typing of *S. aureus* isolates by PCR-ribotyping method 1

Type	Isolate*	No. of isolates
1	2A, 2B, 3A, 4, 5, 6, 7A, 7B, 8, 31174, 31175, 31176, 31177, 31179, 31180, 31181, 31182, 31183, 31036, E, SV6, SV289, PD486, PD458, PD380, PD27, 24, 34, 38, 77, 85, 99, 100, 105, 122, 123, 564, 572, 582, 596, 3, 11, 20, 25, 30, 48, 51, 52, 53, 88, 170, 172, 259, 329,330, 352, 373, 390, 410, 451, 31, 33, 47, [a]HUM-2, [a]HUM-3	65
2	35, 36, 98, 104, 580, 595, 597, 598	8
3	82, 83, 86, 562	4
4	199, 214	2
5	496, 525	2
6	217	1
7	235	1
8	31178	1
9	32528	1
10	K	1
11	NFR	1
12	31039	1
13	[a]CN4575, [a]8530	2
14	[a]HUM-11, [a]HUM-G3	2
15	[a]RN4220	1
16	[a]HUM-108	1
17	[a]HUM-284	1
18	[a]HUM-294	1
19	[b]BOV-65	1
20	[b]BOV-66	1
21	[b]BOV-67	1
22	[b]BOV-32276	1
23	[c]ZM-7	1
24	[d]HOR-31912	1

* All are avian isolates except:
[a]: Human isolate
[b]: Bovine isolate
[c]: Zoo mouse isolate
[d]: Horse isolate

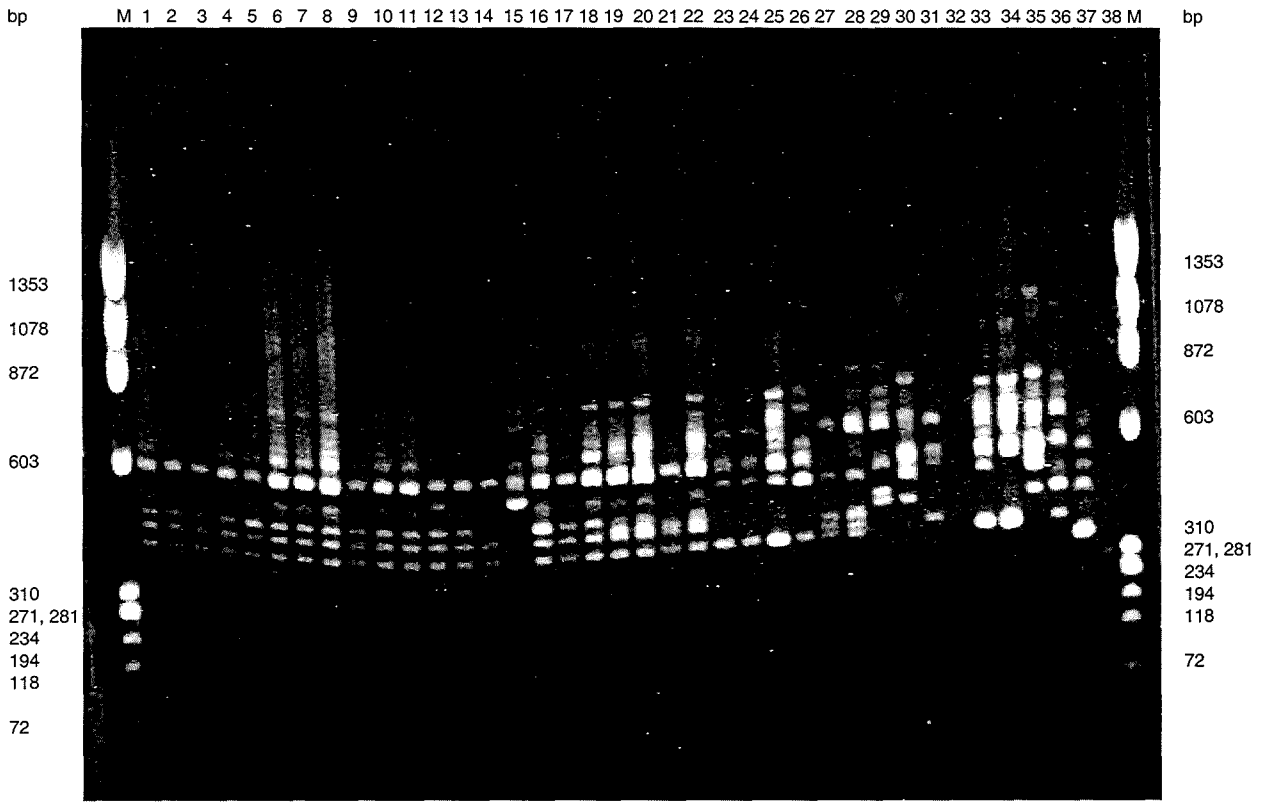


Figure 3.12 Examples of fingerprints obtained by PCR-ribotyping (method 1). Lanes M: ϕX174 RF DNA/Hae III Fragments DNA Ladder. Lanes 1-34: (except lane 32 is negative control) avian *S. aureus* isolates, 24, 11, 3A, 4, 6, 7A, 31175, 31179, PD486, PD27, 31036, 31039, E, K, NFR, SV6, SV289, 34, 595, 104, 35, 98, 82, 83, 86, 562, 496, 525, 31178, 32528, 214, 217 and 235 respectively. Lanes 35 and 36: human *S. aureus* isolates, CN4575 and RN4220 respectively. Lane 37: human *S. epidermidis* isolate, 134. Lane 38: avian *S. cohnii* isolate, 31037. The profiles have been arranged so that isolates of the same type are grouped together. Lanes 1-11, 13, 16-18 (type 1), lanes19-22 (type 2), lanes 23-26 (type 3), lane 32 (type 4), lanes 27 and 28 (type 5), lane 33 (type 6), lane 34 (type 7), lane 29 (type 8), lane 30 (type 9), lane 14 (type 10), lane 15 (type 11), lane 12 (type 12), lane 35 (type 13), lane 36 (type 15) (see **Table 3.12**).

With this method a very simple profile was obtained, containing between 1-3 bands ranging from ~0.4-~0.5 kb, but only with twelve of the avian isolates. The other 74 avian *S. aureus* isolates did not show any amplimer with this PCR-ribotyping method. Examples of these results are shown in **Figure 3.13**. Four groups were recognised for the twelve avian *S. aureus* isolates (**Table 3.13**). Group **'a'** included five isolates. Four of the N. Ireland isolates unrelated to PFGE type 24 (according to McCullagh *et al.,* 1998) and one of the American isolates were in this group. Group **'b'** included four isolates, three American and one Scottish isolate. Group **'c'** contained two isolates, which were N. Ireland isolates unrelated to PFGE type 24. Group **'d'** included one Scottish isolate. None of the N. Ireland avian *S. aureus* isolates related to PFGE type 24 or closely-related or possibly related to type 24 and some of unrelated isolates, most of the Scottish isolates, all of the English isolates and all of the Sri Lankan isolates gave a product with this PCR method. Between the 13 human *S. aureus* isolates tested with this method, only isolates HUM-2 and HUM-3 did not show any band by this method. According to the results of PCR-ribotyping method 1, these two human strains had profiles similar to group **1** avian isolates (**Table 3.12**). Some of the human and animal *S. aureus* isolates had bands in common with the avian *S. aureus* strains (**Table 3.13**). None of the avian coagulase-negative, human and animal coagulase-negative isolates gave a product with PCR-ribotyping method 2.

Results obtained with PCR-ribotyping method 2 for the chicken isolates of *S. aureus* from N. Ireland and the other, atypical avian strains not from N. Ireland are included in summary **Tables 3.20** and **3.21**, respectively.

3.5.2 REP-PCR

A REP-PCR has been described for the molecular genotyping of human *S. aureus* isolates (Del Vecchio *et al.,* 1995). This high-stringency PCR fingerprinting method is based on a repetitive sequence found in *Mycoplasma pneumoniae* (Wenzel and Hermann, 1988), but it also generated strain-specific

Table 3.13 Typing of *S. aureus* isolates by PCR-ribotyping method 2

Type[†]	Isolate[*]	No. of isolates
'a'	82, 83, 86, 562, 217	5
'b'	31178, 199, 214, 235, [a]RN4220, [b]BOV-65	6
'c'	496, 525, [a]HUM-11, [a]HUM-G3, [a]HUM-284, [a]HUM-298,[a]HUM-275, [b]BOV-66, [b]BOV-67	9
'd'	32528, [a]CN4575, [a]8530, [c]ZM-7	4
'e'	[a]HUM-BB	1
'f'	[a]HUM-271	1

[†]: See figure 3.13
[*] All are avian isolates except:
[a]: Human isolate
[b]: Bovine isolate
[c]: Zoo mouse isolate

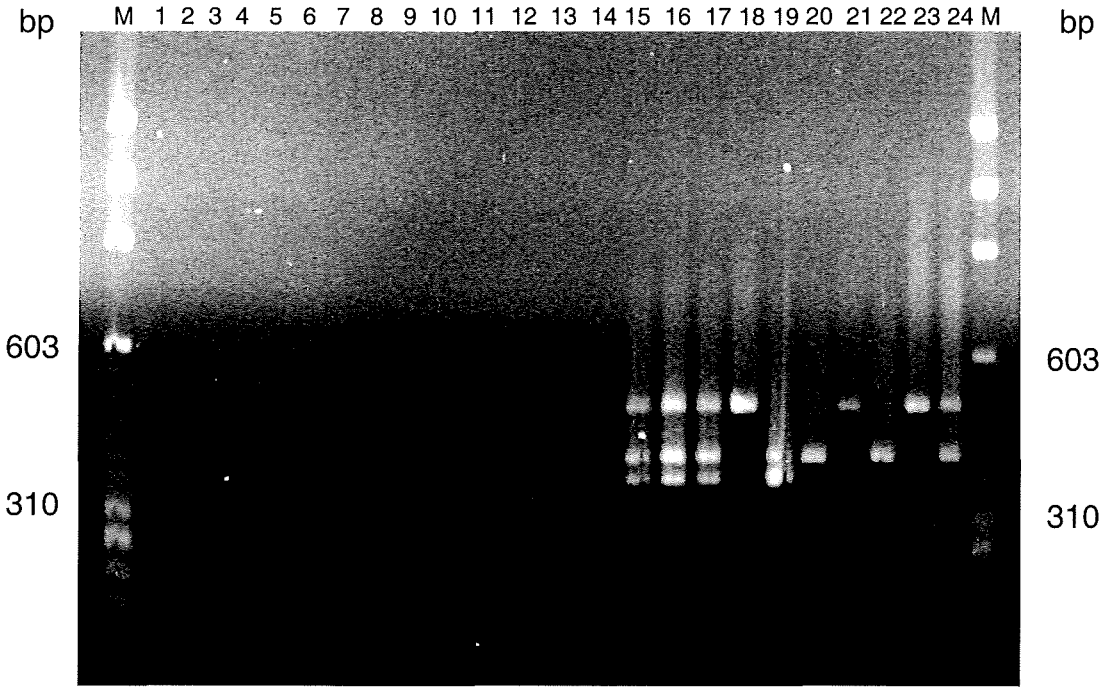


Figure 3.13 Examples of fingerprints obtained by PCR-ribotyping (method 2) for *S. aureus* isolates. Lanes M: ϕX174 RF DNA/Hae III Fragments DNA Ladder. Lanes 1-19: avian *S. aureus* isolates, 24, 77, 85, 99, 100, 105, 122, 123, 34, 38, 104, 35, 36, 98, 82, 83, 86, 496 and 562 respectively. Lanes 20-24: human *S. aureus* isolates, 8530, HUM-11, CN4575, HUM-G3 and RN4220 respectively. Lanes 15-17 and 19 (type 'a'), lane 24 (type 'b'), lanes 18, 21 and 23 (type 'c'), lanes 20 and 22 (type 'd') (see **Table 3.13**).

DNA fragments when human *S. aureus* DNA was used as an amplification template. Primer RW3A (**Table 2.11**) was used as described previously by Del Vecchio *et al.* (1995). The PCR conditions described by Van Der Zee *et al.* (1999) were optimised for use with the avian, human or animal *S. aureus* strains (section 2.6.9.2).

With this method, the profiles of *S. aureus* strains revealed complex patterns containing up to 16 bands ranging from ~0.15-~3 kb with various intensities. Example of these profiles are shown in **Figure 3.14**. Seven groups were recognised for the 86 avian *S. aureus* isolates (**Table 3.14**). Group **A** contained the majority, 67 (80%) of isolates. All N. Ireland *S. aureus* isolates related to PFGE type 24 and closely-related to type 24 and possibly-related to type 24 (according to McCullagh *et al.* 1998) were in group **A**. Most of the Scottish isolates except isolates 31178 and 32528, most of the English isolates except isolate NFR, and all of the Sri Lankan isolates were also in this group. Group **B** contained 6 (7%) isolates. Some of the N. Ireland isolates unrelated to PFGE type 24 were in this group. Group **C** contained five (6%) isolates. Some of the N. Ireland isolates unrelated to PFGE type 24 and one English isolate NFR were in this group. Group **D** contained four (5%) isolates. All of the American isolates were in this group. Group **E** contained two (2%) isolates, both of which were N. Ireland isolates unrelated to PFGE type 24. Groups **F** and **G** contained one (1%) isolate each, from Scottish sources. Of the five human *S. aureus* isolates examined by this method, some of them had patterns similar to the avian *S. aureus* isolates and some of them had different patterns (**Table 3.14** and **Figure 3.14** lanes 24-28).

Results obtained with REP-PCR for the chicken isolates of *S. aureus* from N. Ireland and the other, atypical avian *S.* strains not from N. Ireland are included in summary **Tables 3.20** and **3.21**, respectively.

Table 3.14 Typing of *S. aureus* isolates by REP-PCR

REP-PCR type	Isolate*	No of isolates
A	2A, 2B, 3A, 4, 5, 6, 7A, 7B, 8, 31174, 31175, 31176, 31177, 31179, 31180, 31181, 31182, 31183, 31036, 31039, E, K, SV6, SV289, PD486, PD458, PD380, PD27, 24, 34, 38, 77, 85, 99, 100, 104, 105, 122, 123, 564, 572, 582, 595, 596, 3, 11, 20, 25, 30, 48, 51, 52, 53, 88, 170, 172, 259, 329,330, 352, 373, 390, 410, 451,31, 33, 47	67
B	35, 36, 98, 580, 597, 598	6
C	82, 83, 86, 562, NFR	5
D	199, 214, 217, 235	4
E	496, 525	2
F	32528, [a]CN4575, [a]8530	3
G	31178	1
H	[a]HUM-11, [a]HUM-G3, [a]RN4220	3

* All are avian isolates except for, [a]: Human isolate

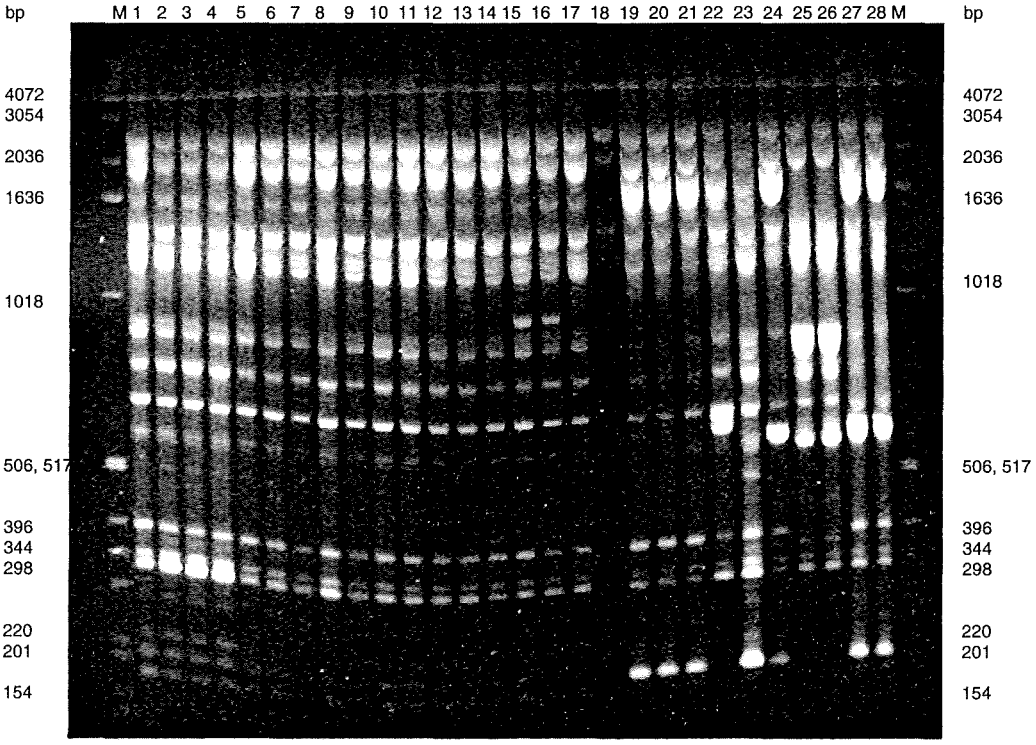


Figure 3.14 Examples of fingerprints obtained by REP-PCR for *S. aureus* isolates. Lanes M: 1 Kb DNA Ladder. Lanes 1-23: avian *S. aureus* isolates, 24, 77, 85, 99, 100, 105, 122, 123, 572, 582, 596, 34, 38, 104, 35, 36, 98, 580, 82, 83, 86, 496 and 562 respectively. Lanes 24-28: human *S. aureus* isolates, HUM-11, 8530, CN4575, HUM-G3 and RN4220 respectively. Lanes 1-14 (type A), lanes 15-18 (type B), lanes 19-21 and 23 (type C), lane 22 (type E), lanes 25-26 (type F), lanes 24 and 27-28 (type H) (see **Table 3.14**).

3.5.3 PCR for coagulase gene

The coagulase protein is an important virulence factor of *S. aureus* encoded by the *coa* gene and the expected size of the amplimer was ~0.4-~0.9 kb. The variable region of *coa* is comprised of 81-bp tandem short sequence repeats (SSRs) (van Belkum *et al.,* 1997) that are variable in both number and sequence (Goh *et al.,* 1992), as determined by restriction fragment length polymorphism (RFLP) analysis of PCR products (Goh *et al.,* 1992; Hookey *et al.,* 1998; 1999). These repeat regions have been used for differentiating human *S. aureus* isolates.

Amplification of the 3′ end of the coagulase gene was done with primers COAG2 and COAG3 (**Table 2.11**) as described previously by Goh *et al.* (1992) for human *S. aureus* isolates. This study modified and optimised the PCR conditions as described by Goh *et al.* (1992), van Belkum *et al.* (1997) and Su *et al.* (1999).

All the 86 avian *S. aureus* isolates produced a single band but with sizes ranging between ~0.6-~0.9 kb (**Figures 3.15a** and **b**). Group **I** included 73 (85%) isolates and the amplimer had a size of ~0.8 kb. All N. Ireland avian *S. aureus* isolates related to PFGE type 24, closely-related to type 24 and possibly-related to type 24 and some of the unrelated strains were in group **I**. This group also included most of the Scottish isolates except isolate 32528 and most of the English isolates except isolates NFR and E and all Sri Lankan isolates. Group **II** contained nine isolates (10%) and the amplimer had a size of ~0.7 kb. Some of the N. Ireland isolates unrelated to PFGE type 24, two American isolates and one English isolate were in this group. Groups **III** include two isolates (2%) and the amplimer had a size of ~0.9 kb. Two American isolates were in this group. Group **IV** contained two isolates (2%) and the size of amplimers was ~0.6 kb. One English and one Scottish isolate were in this group. Some human and animal *S. aureus* isolates showed bands of the same size as the avian *S. aureus* isolates but some of them were different (**Table 3.15**). None of the avian

Table 3.15 PCR amplification of coagulase gene region of *S. aureus* isolates

Coagulase gene type	Isolate*	No. of isolates
I	2A, 2B, 3A, 4, 5, 6, 7A, 7B, 8, 31174, 31175, 31176, 31177, 31178, 31179, 31180, 31181, 31182, 31183, 31036, 31039, K, SV6, SV289, PD486, PD458, PD380, PD27, 24, 34, 35, 36, 38, 77, 85, 98, 99, 100, 104, 105, 122, 123, 564, 572, 580, 582, 595, 596, 597, 598, 3, 11, 20, 25, 30, 48, 51, 52, 53, 88, 170, 172, 259, 329, 330, 352, 373, 390, 410, 451, 31, 33, 47, [a]CN4575, [a]8530	75
II	82, 83, 86, 496, 562, 525, 217, 235, NFR, [a]HUM-11, [a]HUM-G3, [a]HUM-G5, [a]RN4220, [b]BOV-65	14
III	199, 214, [a]HUM-2, [a]HUM-3, [a]HUM-G1, [a]HUM-G2	6
IV	E, 32528	2
V	[c]ZM-7, [b]BOV-32276	2
VI	[d]HOR-31912	1

* All are avian isolates except:
[a]: Human isolate
[b]: Bovine isolate
[c]: Zoo mouse isolate
[d]: Horse isolate

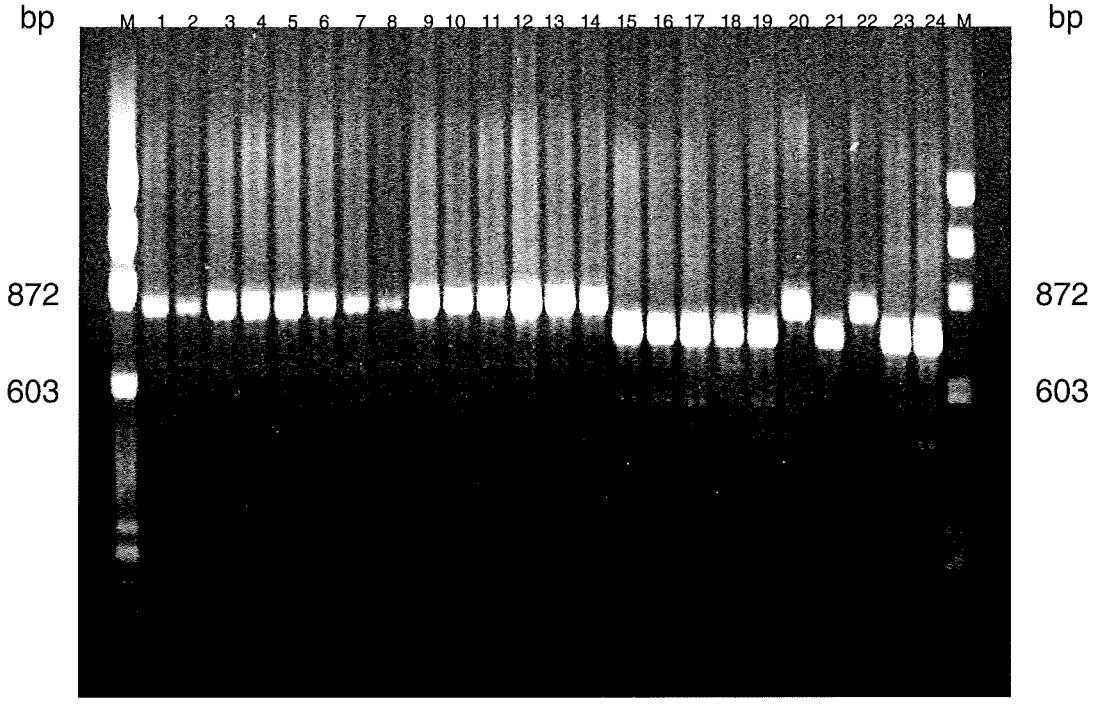


Figure 3.15a Examples of PCR amplification of coagulase gene region for *S. aureus* isolates. Lanes M:ϕX174 RF DNA/Hae III Fragments DNA Ladder. Lanes 1-19: avian *S. aureus* isolates, 24, 77, 85, 99, 100, 105, 122, 123, 34, 38, 104, 35, 36, 98, 82, 83, 86, 496 and 562 respectively. Lanes 20-24: human *S. aureus* isolates, 8530, HUM-11, CN4575, HUM-G3 and RN4220 respectively. Lanes 1-14, 20 and 22 (type I), Lanes 15-19, 21, 23 and 24 (type II) (see **Table 3.15**).

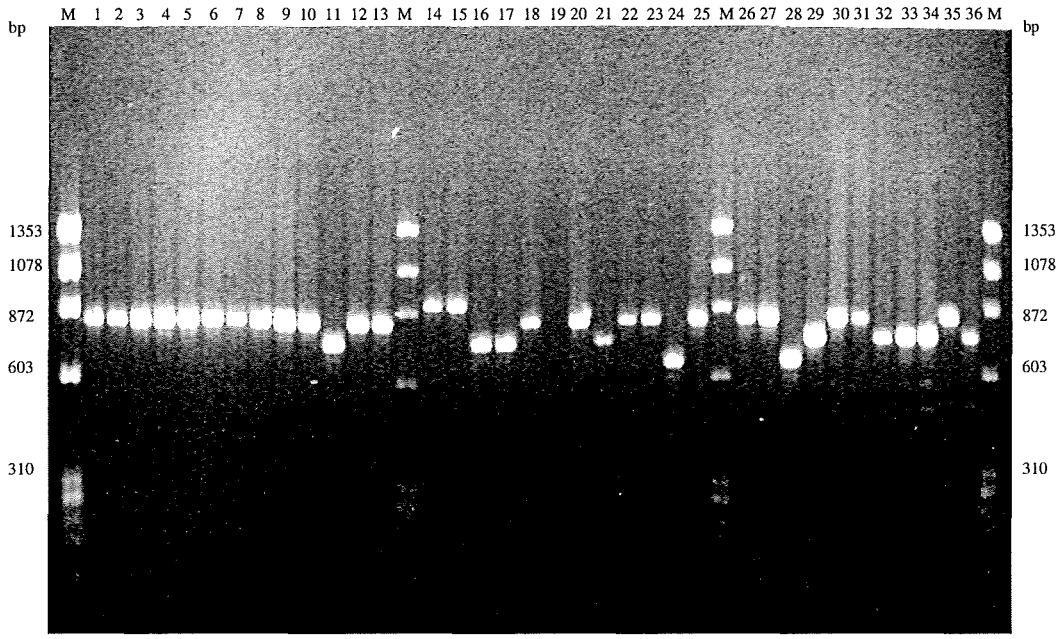


Figure 3.15b Examples of PCR amplification of coagulase gene region for *S. aureus* isolates. Lanes M: ϕX174 RF DNA/Hae III Fragments DND Ladder. Lanes 1-18, 20-28 and 30-34: avian *S. aureus* isolates 24, 564, 595, 580, 597, 598, 11, 31, PD486, 47, 525, 85, PD27, 199, 214, 217, 235, SV6, SV289, NFR, 31036, 31039, E, K, 31174, 31178, 32528, 104, 98, 82, 496, and 562 respectively. Lane 19: avian *S. cohnii* isolate 31037 (negative control). Lane 29: bovine *S. aureus* isolate BOV-65. Lanes 35 and 36: human *S. aureus* isolates CN4575 and RN4220 respectively. Lanes 1-10, 12-13, 18, 20, 22-23, 25-27, 30-31 and 35 (type I), lanes 11, 16-17, 21, 29 and 32-34 (type II), lanes 14-15 (type III), lanes 24 and 28 (type IV) (see **Table 3.15**).

coagulase-negative isolates showed an amplimer with the coagulase gene primers (**Figures 3.15a** and **b**).

Results obtained with the coagulase gene region for the chicken isolates of *S. aureus* from N. Ireland and the other, atypical avian strains not from N. Ireland are included in summary **Tables 3.20** and **3.21**, respectively.

3.5.3.1 Restriction enzyme digestion of the PCR product of the coagulase gene

In order to further improve the discrimination between avian and other isolates of *S. aureus* by the various PCR fingerprinting methods, some of the PCR products from analyses where only 1 or 2 bands fragments were produced were treated with particular restriction enzyme and the resulting patterns analysed.

With the coagulase gene amplimer, the 50 avian *S. aureus* isolates that were investigated produced a number of different restriction patterns when *AluI* (Goh *et al.,* 1992) was used. The fragments sizes ranged from ~0.1-~0.6 kb (e.g. see **Figure 3.16**). On the basis of the patterns, eight groups were recognised for the 50 isolates and each was assigned a letter (lower case) (**Table 3.16**). Group **'aa'** included 28 (56%) of the isolates. All the N. Ireland avian *S. aureus* related to PFGE type 24, closely-related to type 24 and also most of Scottish isolates except for 31178 and 32528, all Sri Lankan isolates and some of the English isolates tested were in this group. Group **'bb'** included eight (16%) isolates. Some of the N. Ireland strains unrelated to PFGE type 24 were in this group. Group **'cc'** included seven (14%) isolates. Some of the N. Ireland unrelated to PFGE type 24, two American and one English isolate were in this group. Group **'dd'** and **'ee'** each included two (4%) isolates and groups **'ff'**, **'gg'** and **'hh'** each contained one (2%) isolate.

Comparison of the coagulase gene restriction fragment length polymorphism (RFLP) patterns with PCR results for the coagulase gene revealed that the former method was much more discriminating than the latter. For example

Table 3.16 Restriction analysis of the PCR product of the coagulase gene of *S. aureus* isolates by *AluI*

Type	Isolate*	No. of isolates
aa	5, 7A, 31174, 31036, 31039, K PD458, PD380, PD27, SV6, SV289, 24, 11, 77, 85, 99, 100, 105, 122, 123, 3, 20, 31, 33, 47, 34, 38, 564	28
bb	595, 104, 35, 36, 98, 580, 597, 598	8
cc	82, 83, 86, 562, 217, 235, NFR	7
dd	496, 525	2
ee	199, 214	2
ff	32528, [a]RN4220, [a]HUM-11, [a]HUM-G3	4
gg	31178	1
hh	E	1
ii	[a]8530, [a]CN4575	2

* All are avian isolates except:
[a]: Human isolate

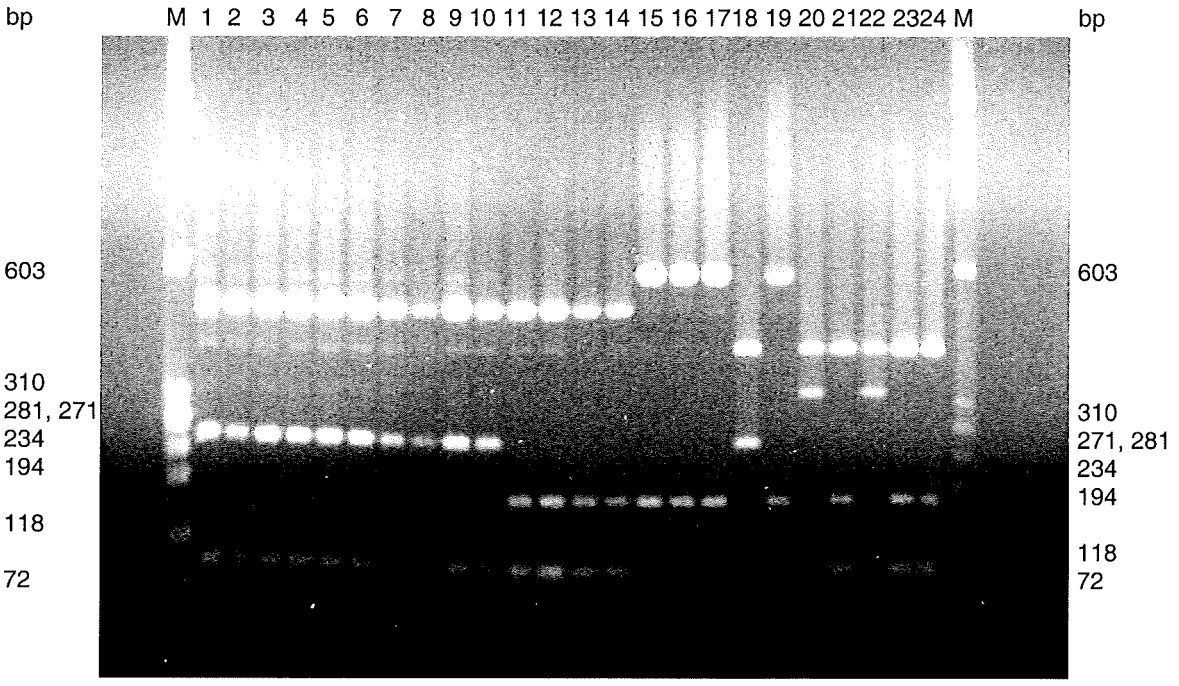


Figure 3.16 Examples of band patterns obtained for *S. aureus* isolates after PCR amplification of coagulase gene region and restriction with *AluI* . Lanes M: ϕX174 RF DNA/Hae III Fragments DNA Ladder. Lanes 1-19: avian *S. aureus* isolates, 24, 77, 85, 99, 100, 105, 122, 123, 34, 38, 104, 35, 36, 98, 82, 83, 86, 496 and 562 respectively. Lanes 20-24: human *S. aureus* isolates, 8530, HUM-11, CN4575, HUM-G3 and RN4220 respectively. Lanes 1-10 (type aa), lanes 11-14 (type bb), lanes 15-17 and 19 (type cc), lane 18 (type dd), lanes 20 and 22 (type ii), lanes 21, 23 and 24 (type ff) (see **Table 3.16**).

isolate 31178 by the PCR method had a band similar to that of isolates in group **'aa'** (**Table 3.15**) but by RFLP had a different pattern (**Table 3.16**). Similarly, isolates 35, 36, 98, 104, 595, 580, 597 and 598 were indistinguishable from other isolates in group **'aa'** by PCR but, after restriction by *AluI,* they had distinct patterns (**Table 3.16**). Isolates 496 and 525 grouped with isolates **'cc'** by PCR but, after restriction of the coagulase gene, they had different patterns (**Table 3.16**). Isolates E and 32528 were identical to each by PCR, but again, after restriction, they had different patterns (**Table 3.16**). Also, some of the human *S. aureus* isolates after restriction showed differences that were not apparent from the PCR product of the coagulase gene (**Tables 3.15, 3.16** and **Figure 3.16**).

Results obtained with restriction of the PCR product of the coagulase gene region for the chicken isolates of *S. aureus* from N. Ireland and the other, atypical avian strains not from N. Ireland are included in summary **Tables 3.20** and **3.21**, respectively

3.5.4 PCR for X-region of the protein A gene

Protein A is another virulence factor of *S. aureus* and forms an immunoglobulin-binding capsule around the bacterium. Briefly the repetitive X-region within the gene of expected size ~0.3- ~0.6 kb was amplified with oligonucleotide primers PROTA1 and PROTA2 (**Table 2.11**) as described by Frenay *et al.* (1994). This study modified and optimised the PCR conditions described previously Frenay *et al.* (1994) for human *S. aureus* isolates.

PCR for the protein A gene of the 86 avian *S. aureus* isolates gave a single band ranging between ~0.3-~0.6 kb (**Figure 3.17**). The avian isolates could be differentiated into five groups. Group **'A'**, contained the majority of 72 (83%) isolates. All of the N. Ireland avian *S. aureus* related to PFGE type 24, closely-related to type 24, possibly-related to type 24 and some of unrelated to type 24 according to McCullagh *et al.* (1998) were in group **'A'**. Most of Scottish isolates except for 31178, 31036 and 32528 and all Sri Lanka isolates and some

Table 3.17 PCR amplification of X-region of the protein A gene of *S. aureus* isolates

Protein A gene type	Isolate*	No. of isolates
'A'	2A, 2B, 3A, 4, 5, 6, 7A, 7B, 8, 31174, 31175, 71176, 31177, 31179, 31180, 31181, 31182, 31183, 31039, E, SV289, PD486, PD458, PD380, PD27, 235, 24, 34, 35, 36, 38, 77, 85, 98, 99, 100, 104, 105, 122, 123, 562, 564, 572, 580, 582, 595, 596, 597, 598, 3, 11, 20, 25, 30, 48, 51, 52, 53, 88, 170, 172, 259, 329, 330, 352, 373, 390, 410, 451, 31, 33, 47, [a]HUM-2, [a]CN4575, [a]HUM-11, [a]8530, [a]HUM-G3, [a]HUM-G4	78
'B'	82, 83, 86, 199, 214, 217, NFR	7
'C'	32528, 496, 525, [a]HUM-3, [a]HUM-G2, [b]HOR-31912	6
'D'	SV6, K, [a]HUM-G5, [a]RN4220, [c]ZM-7	4
'E'	31178, 31036, [a]HUM-G1, [a]HUM-G6	4
'F'	[d]BOV-32276	1
'G'	[d]BOV-65	1

* All are avian isolates except:
[a]: Human isolate
[b]: Horse isolate
[c]: Zoo mouse isolate
[d]: Bovine isolate

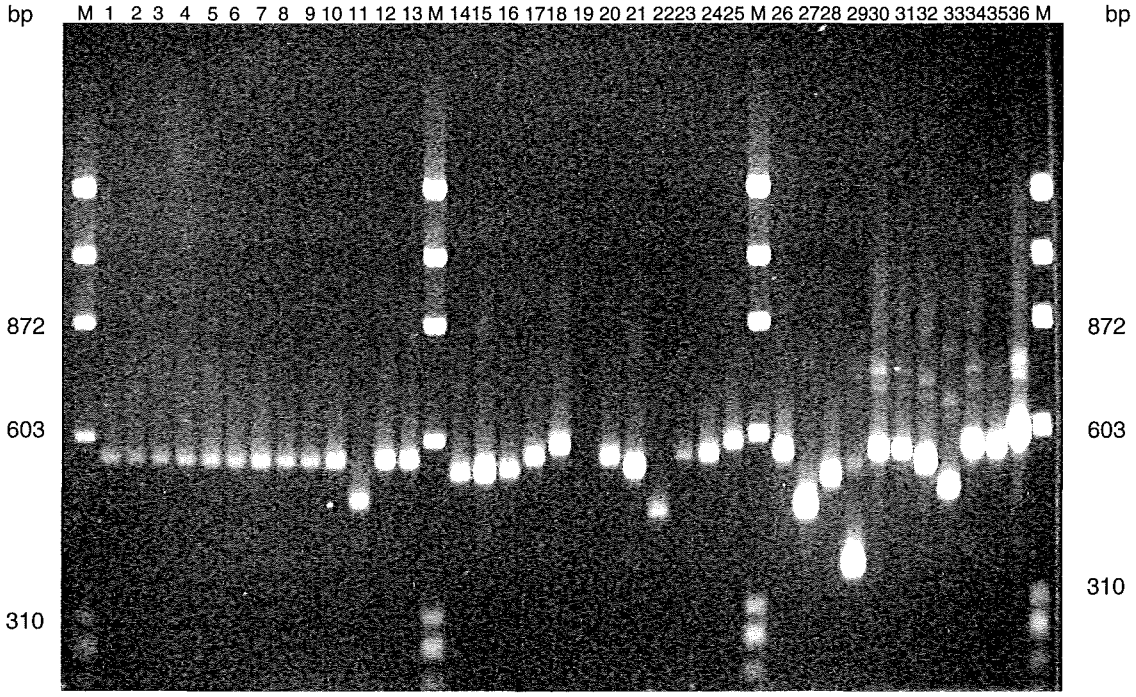


Figure 3.17 Examples of PCR amplification of X-region of the protein A gene for *S. aureus* isolates. Lanes M: ϕX174 RF DNA/Hae III Fragments DND Ladder. Lanes 1-18, 20-28 and 30-34: avian *S. aureus* isolates 24, 564, 595, 580, 597, 598, 11, 31, PD486, 47, 525, 85, PD27, 199, 214, 217, 235, SV6, SV289, NFR, 31036, 31039, E, K, 31174, 31178, 32528, 104, 98, 82, 496, and 562 respectively. Lane 19: avian *S. cohnii* isolate 31037. Lane 29: bovine *S. aureus* isolate BOV-65. Lanes 35 and 36: human *S. aureus* isolates CN4575 and RN4220 respectively. Lanes 1-10, 12-13, 17, 20, 23-24, 26, 30-31 and 34-35 (type 'A'), lanes 14-16, 21 and 32 (type 'B'), lanes 11, 28 and 33 (type 'C'), lanes 18, 25 and 36 (type 'D'), lanes 22 and 27 (type 'E'), lane 29 (type 'G'). (see **Table 3.17**).

English isolates and one American isolate were also in this group (**Table 3.17**). Group **'B'**, contained seven (8%) isolates. Some of the N. Ireland isolates unrelated to type 24 three American isolates and one English isolate were in this group. Group **'C'**, contained three (3%) isolates. Two of the N. Ireland isolates unrelated to type 24 and one Scottish isolate were in this group. Group **'D'** and **'E'** had two (2%) isolates each. Again some of the human and animal *S. aureus* isolates had similar or different bands to the avian isolates (**Table 3.17**). None of the avian, human or animal coagulase-negative isolates produced an amplicon for the protein A gene under these PCR conditions (e.g. lane 19, **Figure 3.17**).

Results obtained with the protein A gene for the chicken isolates of *S. aureus* from N. Ireland and the other, atypical avian strains not from N. Ireland are included in summary **Tables 3.20** and **3.21**, respectively.

3.5.4.1 Restriction of the PCR product of X-region of the protein A gene

RLFP analysis of the protein A gene of 41 avian *S. aureus* isolates with restriction enzyme *RsaI* (van Belkum *et al.,* 1997) identified five different types (**Table 3.18**). The fragments sizes ranged from ~0.1-~0.4 kb (e.g. see **Figure 3.18**). Comparison of the protein A RFLP patterns with PCR results for the protein A gene revealed that they were similar to each other in that RFLP analysis was no more discriminating than the PCR typing method for these isolates. Group **AA** included 27 (66%) isolates, group **BB** contained seven (17%) isolates, group **CC** had three (7%) isolates and groups **DD** and **EE** included two (5%) isolates each. Again, results with four human and a bovine *S. aureus* isolate showed that RFLP was no more discriminating than PCR typing method for protein A (**Table 3.18** and **Figure 3.18**).

Results obtained with restriction of the PCR product of the protein A gene for the chicken isolates of *S. aureus* from N. Ireland and the other, atypical avian strains not from N. Ireland are included in summary **Tables 3.20** and **3.21**, respectively.

Table 3.18 Restriction analysis of PCR product of X-region of the protein A gene of *S. aureus* by *RsaI*

Type	Isolate*	No. of isolates
AA	5, 7A, 31174, 31039, PD4458, PD380, E, 24, 11, 77, 85, 31, 33, 47, 34, 38, 564, 595, 104, 35, 36, 98, 580, 597,598, 562, 235, [a]CN4575, [a]HUM-11,[a]HUM-G3	30
BB	199, 214, 217, 82, 83, 86, NFR	7
CC	32528, 496, 525	3
DD	K, SV6, [a]RN4220	3
EE	31178, 31036	2
FF	[b]BOV-65	1

* All are avian isolates except:
[a]: Human isolate
[b]: Bovine isolate

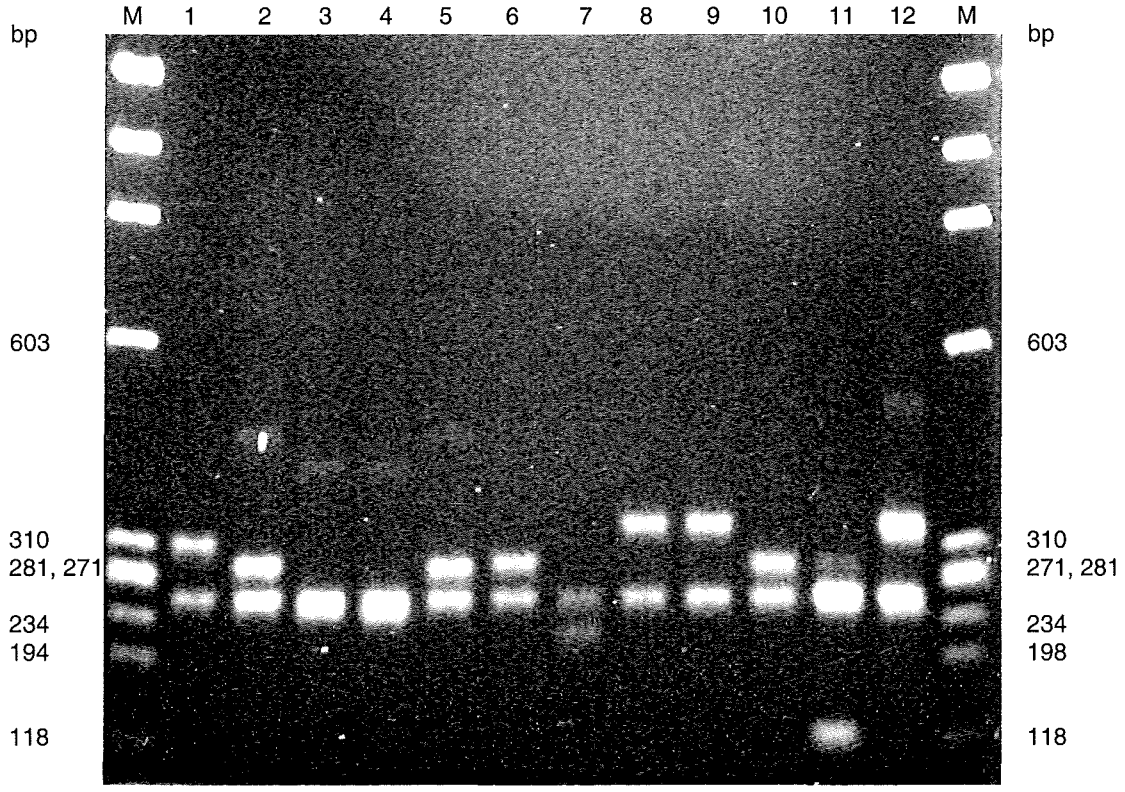


Figure 3.18 Examples of band patterns obtained for *S. aureus* isolates after PCR amplification of X-region of the protein A gene and restriction with *RsaI* . Lanes M: ϕX174 RF DNA/Hae III Fragments DNA Ladder. Lanes 1-10: avian *S. aureus* isolates, 24, 82, 496, 525, 199, 217, 31036, K, SV6 and NFR respectively. Lane 11: bovine *S. aureus* isolate BOV-65. Lane 12: human *S. aureus* isolete RN4220. Lane 1 (type AA), lanes 2, 5-6 and 10 (type BB), lanes 3-4 (type CC), lanes 8-9 and 12 (type DD), lane 7 (type EE), lane 11 (type FF) (see **Table 3.18**).

3.6 CHARACTERISATION OF VIRULENCE-REGULATORY GENES BY PCR

The synthesis of virulence factors and other extracellular proteins by *S. aureus* is globally controlled by the *agr* and *sar* loci. *agr* encodes a two-component signalling pathway whose activating ligand is an *agr*-encoded autoinducing peptide. *sarA* is a DNA-binding protein with binding specificity for the P2 and P3 interpromoter region of *agr*. In this study, three regions of these virulence regulatory genes were targeted, namely *agrP2*, *sarA* and *agrBDC*. (see **Figure 1.1**).

3.6.1 PCR for *agrP2, sarA* and *agrBDC*

The primers used were AGR-1 and AGR-2 for *agrP2*, AGRD123F and AGRD123R for *agrBDC*, SAR-1 and SAR-2 for *sarA* (**Table 2.11**) as described previously by Kornblum *et al.* (1990), (personal communication, Dr. P. McDowell, Institute of Infection and Immunity, The University of Nottingham, Queens Medical Centre, Nottingham), and Chien *et al.* (1998), respectively. In this study the conditions for these three individual PCRs were optimised.

According to these PCR methods, all the 86 avian, human and animal *S. aureus* isolates had a single similar band for each PCR. The fragment size for *agrP2* was 235 bp, for *sarA* was 372 bp and for *agrBDC* was ~1 kb (**Figures 3.19**, **3.20** and **3.21**, respectively). None of the six avian coagulase-negative isolates and an animal *S. intermedius* isolate BOV-32236 and six different species of animal coagulase-negative isolates, when examined by these PCR methods, gave any *agrP2*, *sarA* or *agrBDC* amplimers.

3.6.1.1 Restriction enzyme digestion of the PCR product of agrBDC region

Restriction sites in the sequence for the *agrBDC* region in GenBank (accession number AF001783) were examined using Genejockey II sequence processor (Biosoft, Cambridge, UK). Results showed that *Mse*I cut more frequently than

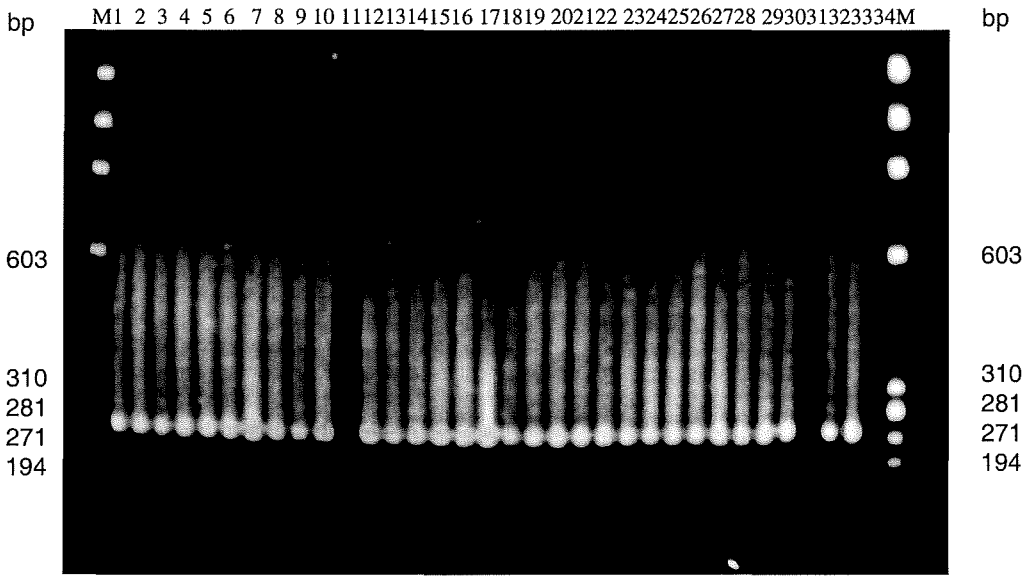


Figure 3.19 Examples of PCR amplification of *agrP2* fragment for *S. aureus* isolates. Lanes M: ϕX174 RF DNA/Hae III Fragments DNA Ladder. Lanes 1-10 and 12-29 avian *S. aureus* isolates 24, 34, 35, 36, 38, 77, 82, 83, 85, 86, 98, 99, 100, 104 105, 122, 123, 496, 562, 564, 572, 580, 582, 595, 596, 597, 2B, SV289 and SV6 respectively. Lane 11: avian *S. cohnii* isolate 31037. Lane 30: avian *S. sciuri* isolate 31040. Lanes 31-32: animal *S. aureus* isolates HOR-31912 and ZM-7 respectively. Lane 33: human *S. aureus* isolate RN4220. Lane 34: animal *S. hominis* isolate DUK-31915.

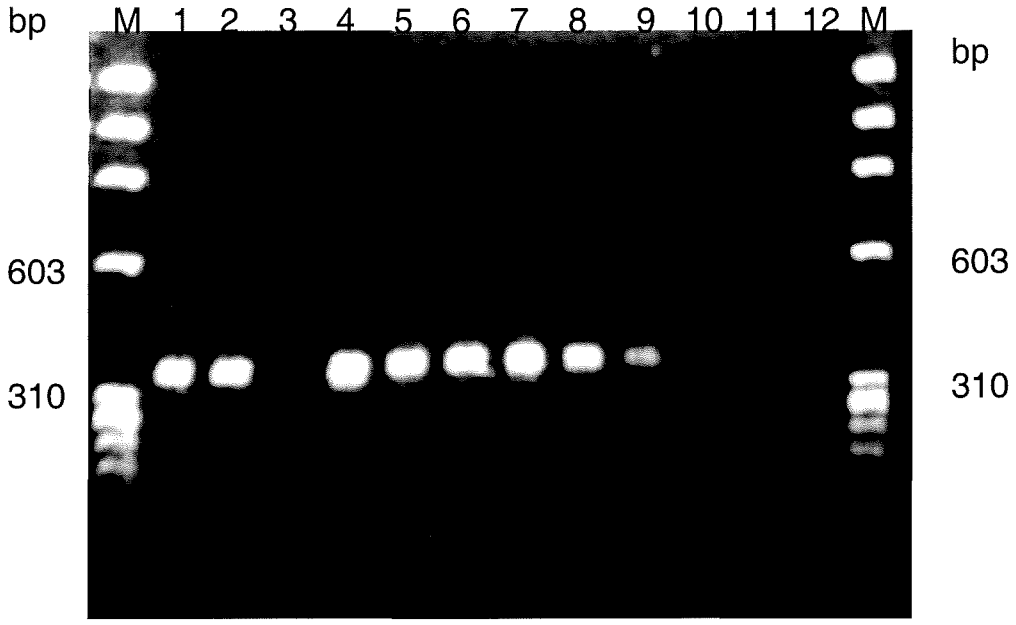


Figure 3.20 Examples of PCR amplification of *sarA* fragment for *S. aureus* isolates. Lanes M: ϕX174 RF DNA/Hae III Fragments DNA Ladder. Lanes 1-2 and 4-6: avian *S. aureus* isolates 2A, 3A, 6, 7B and 31178, respectively. Lanes 3 and 10: avian *S. cohnii* isolates 3B and 31037, respectively. Lanes 7-9: human *S. aureus* isolates HUM-3, 8530 and RN4220, respectively. Lane 12: animal *S. epidermidis* isolate BOV-32304.

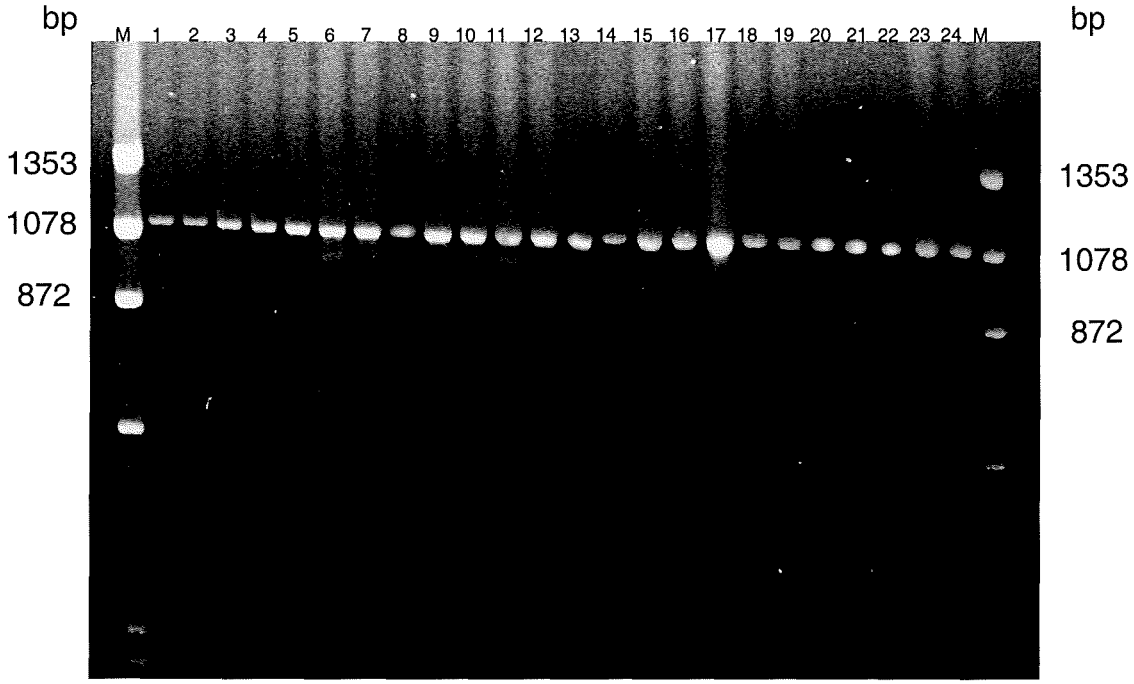


Figure 3.21 Examples of PCR amplification of *agrBDC* region for *S. aureus* isolates. Lanes M: ϕX174 RF DNA/Hae III Fragments DNA Ladder. Lanes 1-19: avian *S. aureus* isolates 24, 77, 85, 99, 100, 105, 122, 123, 34, 38, 104, 35, 36, 98, 82, 83, 86, 496 and 562, respectively. Lanes 20-24: human *S. aureus* isolates 8530, HUM-11, CN4575, HUM-G3 and RN4220, respectively.

other enzymes, so this enzyme was selected for restriction analysis of the PCR products of the *agrBDC* region.

MseI was used to digest the PCR products from the *agrBDC* region of 39 avian *S. aureus* isolates. The size of the restriction fragments ranged from ~100 to ~300 bp (e.g. see **Figure 3.22**). The isolates could be differentiated into three groups (**Table 3.19**). Most isolates were fitted into group **'II'** that included 28 (72%) isolates. Isolates in this group included the N. Ireland strains related to PFGE type 24, and closely-related, possibly-related, and some unrelated strains. Also included were the Scottish, English, American and Sri Lankan isolates. Group **'I'** contained seven (18%) isolates. Isolates this group included some of the N. Ireland strains unrelated to PFGE type 24, two of the American and one of the English isolates. Group **'III'** included four (10%) isolates. Isolates in this group included two of the N. Ireland strains unrelated to PFGE type 24 and two of the Scottish isolates. Of the four human *S. aureus* samples restricted by *MseI*, two isolates were included in each of groups **'I'** and **'III'** (**Figure 3.22** and **Table 3.19**).

Results obtained with restriction of the PCR product of the *agrBDC* region for the chicken isolates of *S. aureus* from N. Ireland and the other, atypical avian strains not from N. Ireland are included in summary **Tables 3.20** and **3.21**, respectively.

3.7 TOXIN DETECTION

Prior to this study, there was little published information on which toxins, if any, are produced by avian isolates of *S. aureus*. This was investigated by molecular methods, PCR amplification of toxin genes, and by biological assay.

Table 3.19 Restriction analysis of PCR product from *agrBDC* region of *S. aureus* by *MseI*

Type	Isolate*	No. of isolates
'I'	82, 83, 86, 562, NFR, 217, 235, [a]HUM-G3, [a]RN4220	9
'II'	2A, 2B, 5, 7A, 7B, 31174, 31036, 31039, E, K, PD27, PD458, PD380, 199, 214, 24, 77, 85, 99, 100, 122, 123, 34, 38, 595, 104, 35, 98	28
'III'	496, 525, 31178, 32528, [a]CN4575, [a]8530	6

* All are avian isolates except for, [a]: Human isolate

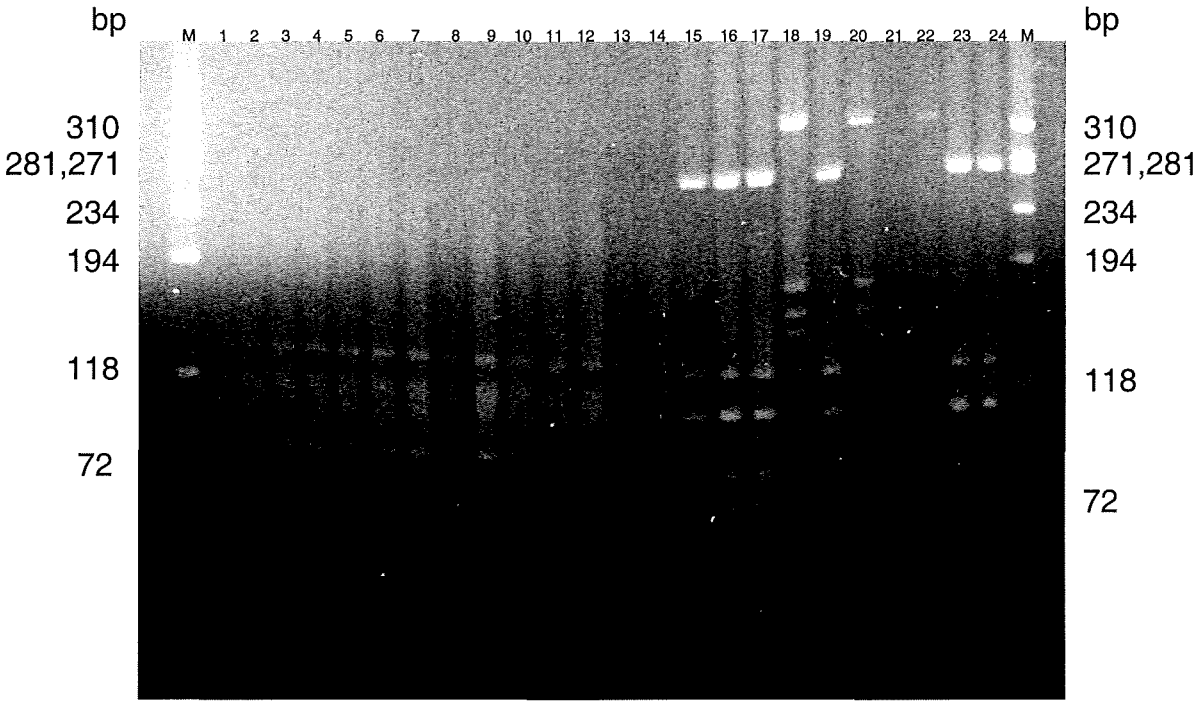


Figure 3.22 Examples of band patterns obtained for *S. aureus* isolates after PCR amplification of *agrBDC* region and restriction with *MseI*. Lanes M: ϕX174 RF DNA/Hae III Fragments DNA Ladder. Lanes 1-19: avian *S. aureus* isolates, 24, 77, 85, 99, 100, 105, 122, 123, 34, 38, 104, 35, 36, 98, 82, 83, 86, 496 and 562 respectively. Lanes 20-24: human *S. aureus* isolates, 8530, HUM-11, CN4575, HUM-G3 and RN4220 respectively. Lanes 1-14 (type 'II'), lanes 15-17, 19, 23-24 (type 'I'), lanes 18, 20 and 22 (type 'III') (see **Table 3.19**).

Table 3.20 Summary of results of genotyping fingerprinting methods for chicken isolates of *S. aureus* from N. Ireland

No.	Number of isolate	PFGE type	Biotype	PCR-ribotyping method 1	PCR-ribotyping method 2	REP-PCR	PCR for coagulase region	Restriction of coagulase region (*AluI*)	PCR for protein A region	Restriction of protein A region (*RsaI*)	Restriction of 1 kb *agrBDC* region (*MseI*)	Haemolysin genes
1	24	24	Poultry	1	0 (No band)	A	I	aa	'A'	AA	'I'	α, δ, γ
2	77	24	Poultry	1	0	A	I	aa	'A'	AA	'I'	α, δ, γ
3	85	24	Poultry	1	0	A	I	aa	'A'	AA	'I'	α, δ, γ
4	99	24	Poultry	1	0	A	I	aa	'A'	/	'I'	α, δ, γ
5	100	24	Poultry	1	0	A	I	aa	'A'	/	'I'	α, δ, γ
6	105	24	Poultry	1	0	A	I	aa	'A'	/	'I'	α, δ, γ
7	122	24	Poultry	1	0	A	I	aa	'A'	/	'I'	α, δ, γ
8	123	24	Poultry	1	0	A	I	aa	'A'	/	'I'	α, δ, γ
9	572	24	Poultry	1	0	A	I	aa	'A'	/	/	α, δ, γ
10	582	24	Poultry	1	0	A	I	aa	'A'	/	/	α, δ, γ
11	596	24	Poultry	1	0	A	I	aa	'A'	/	/	α, δ, γ
12	3	24	Poultry	1	0	A	I	aa	'A'	/	/	α, δ, γ
13	20	24	Poultry	1	0	A	I	aa	'A'	/	/	α, δ, γ
14	25	24	Poultry	1	0	A	I	/	'A'	/	/	α, δ, γ
15	30	24	Poultry	1	0	A	I	/	'A'	/	/	α, δ, γ
16	48	24	Poultry	1	0	A	I	/	'A'	/	/	α, δ, γ
17	51	24	Poultry	1	0	A	I	/	'A'	/	/	α, δ, γ
18	52	24	Poultry	1	0	A	I	/	'A'	/	/	α, δ, γ
19	53	24	Poultry	1	0	A	I	/	'A'	/	/	α, δ, γ
20	88	24	N/T	1	0	A	I	/	'A'	/	/	α, δ, γ
21	170	24	N/T	1	0	A	I	/	'A'	/	/	α, δ, γ
22	172	24	N/T	1	0	A	I	/	'A'	/	/	α, δ, γ
23	259	24	N/T	1	0	A	I	/	'A'	/	/	α, δ, γ
24	329	24	N/T	1	0	A	I	/	'A'	/	/	α, δ, γ
25	330	24	N/T	1	0	A	I	/	'A'	/	/	α, δ, γ
26	352	24	N/T	1	0	A	I	/	'A'	/		α, δ, γ
27	373	24	N/T	1	0	A	I	/	'A'	/	/	α, δ, γ
28	390	24	N/T	1	0	A	I	/	'A'	/	/	α, δ, γ
29	410	24	N/T	1	0	A	I	/	'A'	/	/	α, δ, γ
30	451	24	N/T	1	0	A	I	/	'A'	/	/	α, δ, γ
31	11	24	NHS	1	0	A	I	aa	'A'	AA	'I'	α, δ, γ
32	31	24	NHS	1	0	A	I	aa	'A'	AA	'I'	α, δ, γ
33	33	24	NHS	1	0	A	I	aa	'A'	AA	'I'	α, δ, γ
34	47	24	NHS	1	0	A	I	aa	'A'	AA	'I'	α, δ, γ
35	34	CR	NHS	1	0	A	I	aa	'A'	AA	'I'	α, δ, γ
36	38	CR	Poultry	1	0	A	I	aa	'A'	AA	'I'	α, δ, γ
37	564	CR	Poultry	1	0	A	I	aa	'A'	AA	'I'	α, δ, γ
38	595	CR	Poultry	2	0	A	I	bb	'A'	AA	'I'	α, δ, γ
39	104	PR	Poultry	2	0	A	I	bb	'A'	AA	'I'	α, δ, γ
40	35	UR	Poultry	2	0	B	I	bb	'A'	AA	'I'	α, δ, γ
41	36	UR	Poultry	2	0	B	I	bb	'A'	AA	'I'	α, δ, γ
42	98	UR	Poultry	2	0	B	I	bb	'A'	AA	'I'	α, δ
43	580	UR	Poultry	2	0	B	I	bb	'A'	AA	'I'	α, δ, γ
44	597	UR	Poultry	2	0	B	I	bb	'A'	AA	'I'	α, δ, γ
45	598	UR	Poultry	2	0	B	I	bb	'A'	AA	'I'	α, δ, γ
46	82	UR	Poultry	3	'a'	C	II	cc	'B'	BB	'II'	α, δ
47	83	UR	Poultry	3	'a'	C	II	cc	'B'	BB	'II'	α, δ
48	86	UR	Poultry	3	'a'	C	II	cc	'B'	BB	'II'	α, δ
49	562	UR	Slaughter	3	'a'	C	II	cc	'A'	AA	'II'	α, δ, (β)
50	496	UR	NHS	5	'c'	E	II	dd	'C'	CC	'III'	α, δ, β
51	525	UR	NHS	5	'c'	E	II	dd	'C'	CC	'III'	α, δ, β

I 24= Isolate type 24, CR= Closely related to isolate type 24, PR= Possibly related to isolated type 24, UR= Unrelated to isolate type 24, NHS= Non host specific biotype, Slaughter= Slaughterhouse biotype, / = Not done, N/T= Not biotyped. α= Alpha-haemolysin, δ= Delta haemolysin, γ= Gamma haemolysin, β= Beta haemolysin, (β)= Beta haemolysin detected by biological method but not by PCR for toxin gene. Isolates 496 and 525 produced enterotoxin A

Table 3.21 Summary of results of genotyping fingerprinting methods for atypical avian *S. aureus* isolates not from N. Ireland

No.	Designation of isolate	Source	Geographical origion	PCR-ribotyping method 1	PCR-ribotyping method 2	REP-PCR	PCR for coagulase region	Restriction of coagulase region (*AluI*)	PCR for protein A region	Restriction of protein A region (*RsaI*)	Restriction of 1 kb *agrBDC* region (*MseI*)	Haemolysin gene
1	31036	Chick.	Scotland	1	0	A	I	aa	'E'	EE	'I'	α, δ, γ
2	31039	Chick.	Scotland	12	0	A	I	aa	'A'	AA	'I'	α, δ, (β)
3	E	Gosh.	England	1	0	A	IV	hh	'A'	AA	'I'	α, δ, γ
4	K	Saker	England	10	0	A	I	aa	'D'	DD	'I'	α, δ, γ
5	NFR	Gyr F.	England	11	0	C	II	cc	'B'	BB	'II'	α, δ
6	31178	Chick.	Scotland	8	'b'	G	I	gg	'E'	EE	'III'	α, δ, γ
7	32528	Cock.	Scotland	9	'd'	F	IV	ff	'C'	CC	'III'	α, δ, γ, β
8	199	Duck	USA	4	'b'	D	III	ee	'B'	BB	'I'	α, δ, γ
9	214	Duck	USA	4	'b'	D	III	ee	'B'	BB	'I'	α, δ, γ
10	217	Duck	USA	6	'a'	D	II	cc	'B'	BB	'II'	α, δ
11	235	Duck	USA	7	'b'	D	II	cc	'A'	AA	'II'	α, δ

Chick.= Chicken, Gosh.= Goshawk, Saker= Saker Falcon, Cock.= Cockatoo, Gyr F.= Gyr Falcon, α= Alpha-haemolysin, δ= Delta haemolysin, γ= Gamma haemolysin, β= Beta haemolysin, (β)= Beta haemolysin detected by biological method but not by PCR for toxin gene, isolate 31178 produced enterotoxin B, isolate 32528 produced enterotoxin A, toxic shock syndrome toxin-1 (TSST-1) and exfoliative toxin B

3.7.1 Molecular methods

3.7.1.1 Multiplex PCR for enterotoxins A-E, exfoliative toxins A-B and toxic shock syndrome toxin-1 (TSST-1)

When serious staphylococcal infections occur there is an essential need for rapid and specific detection of the toxins that are present. Conventional methods such as immunodiffusion, enzyme-linked immunosorbent assay (ELISA), and reverse passive agglutination are commonly used, where the actual toxins are identified using antibodies. However, this approach has disadvantages in that it may take too long to get the results, as in the ELISA, or more importantly, the tests do not detect low concentrations of toxin or the toxins may not be detected because of the presence of interfering bacterial products so giving false negative results. On the other hand, with the use of PCR, it is the genes that encode the toxin that are detected. Multiplex PCR allows for the detection of several genes in the one test and, in this study, one multiplex PCR assay was developed to identify the genes of the eight toxins. At the time of this study, there was no report of a multiplex PCR able to amplify these eight toxin genes in a single PCR reaction. The reaction conditions for the multiplex PCR assay were optimised to ensure that all of the target gene sequences were satisfactorily amplified. The primers used were SEA-1 and SEA-2 for enterotoxin A, SEB-3 and SEB-4 for enterotoxin B, SEC-3 and SEC-4 for enterotoxin C, SED-3 and SED-4 for enterotoxin D, SEE-3 and SEE-2 for enterotoxin E, ETA-1 and ETA-2 for exfoliative A, ETB-1 and ETA-2 for exfoliative B and TST-3 and TST-6 for toxic shock syndrome toxin-1 (**Table 2.11**) as described by Johnson *et al.* (1991); Sakurai *et al.* (1995); Becker *et al.* (1998); and personal communication (Prof. C. G. Gemmell, Department of Bacteriology, Glasgow Royal Infirmary).

The individual primer pairs used for the amplification of regions in the eight toxin genes were first tested with their corresponding bacterial control cultures and all gave positive reactions, the PCR products for the genes being present at the correct size. These individual reactions were then combined into a multiplex PCR assay and an example of the results with the control preparation, a mixture

of DNA from known positive strains, is shown in **Figure 3.23** lanes C. When this multiplex PCR method was applied to the 86 avian *S. aureus* strains, only four isolates (5%) gave an amplimer with primers for one or more of the toxins genes. Isolate 31178 gave an amplimer for the enterotoxin B gene, isolate 32528 had three toxin genes: those for TSST-1 (toxic shock syndrome toxin-1), enterotoxin A and exfoliative toxin B and two isolates 496 and 525 gave an amplimer for the enterotoxin A gene (**Figure 3.23**). All of these isolates had shown bands with the PCR-ribotyping method 2 and were, therefore, not considered to be typical avian strains. The sizes of the amplimers for all of these isolates were similar to those obtained with the human strains and were of the expected size (**Table 3.22**). Of the 17 human *S. aureus* isolates examined for these toxins, nine isolates (53%) produced amplimers for different toxin genes: Isolates HUM-2 and HUM-271 (enterotoxin A), isolate HUM-G1 (exfoliative toxin A), isolate HUM-G2 (exfoliative toxin B), isolate HUM-G3 (enterotoxins A and B), isolate HUM-G4 (enterotoxin D), isolate HUM-G5 (enterotoxin E), isolate HUM-G6 (enterotoxin C and TSST-1), isolate CN4575 (enterotoxin B). Human isolates RN4220, HUM-11, 8530, HUM-275, HUM-294, HUM-298 and HUM-BB did not produce any toxins amplimers. Of the six animal *S. aureus* examined by this method, only horse isolate HOR-31912 produced TSST-1, but isolates BOV-32276, ZM-7, BOV-65, BOV-66 and BOV-67 did not produce any toxin amplimers. None of the avian coagulase-negative strain gave products corresponding to any of these toxin genes.

3.7.1.2 Multiplex PCR for α, γ and δ toxin genes

The haemolysins of *S. aureus* are cytotoxic agents which, as a class, may well function to promote invasion of tissues. They can be divided into four classes, namely alpha, beta, delta and gamma haemolysins or toxins.

In this study, a multiplex PCR was developed for the detection of the alpha, delta and gamma toxin genes. The beta toxin gene was detected in a separate

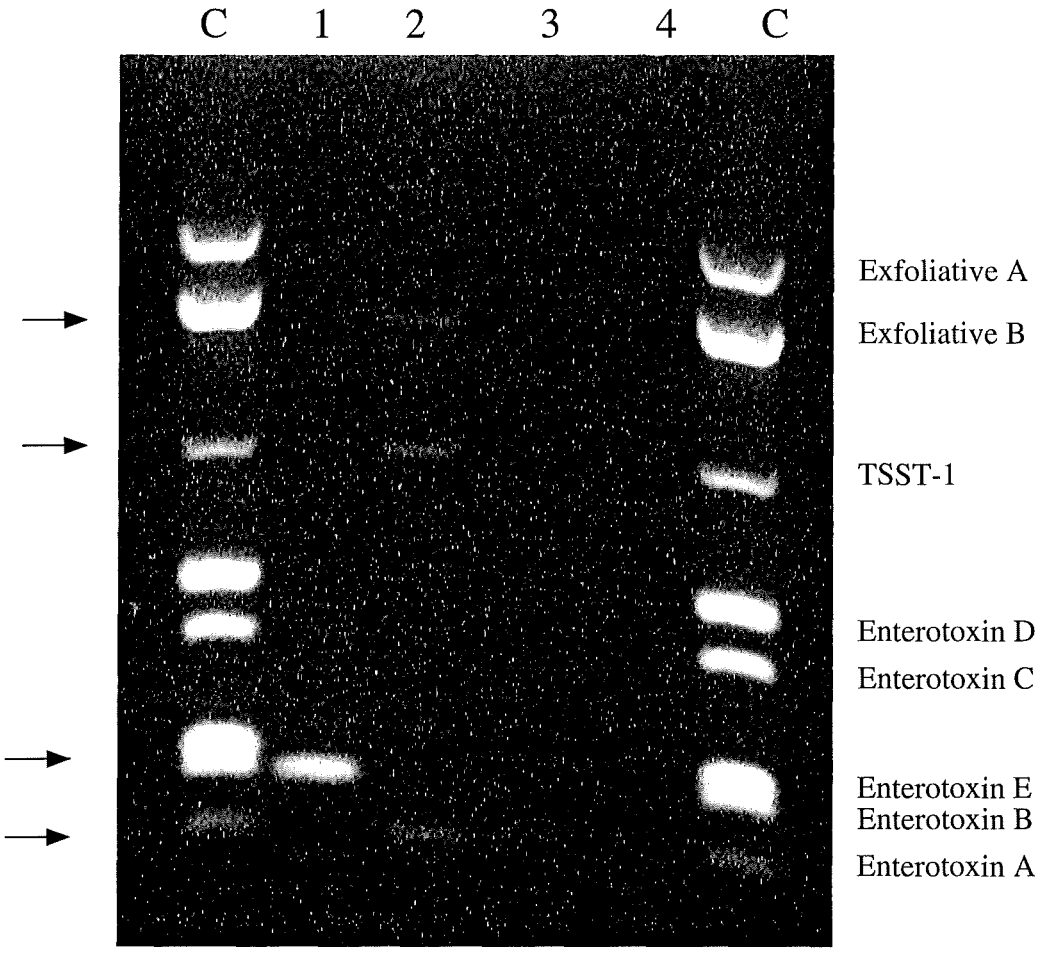


Figure 3.23 Multiplex PCR for enterotoxins A-E, TSST-1 and exfoliative toxins A and B for *S. aureus* isolates. Lane C: Positive control for all eight toxins, lanes 1-4: avian isolates, 31178, 32528, 496 and 2B respectively.

Table 3.22 PCR for staphylococcal toxin genes

Toxin genes	Predicted size (bp) of amplimer
Enterotoxin A (*sea*)	120
Enterotoxin B (*seb*)	163
Enterotoxin C (*sec*)	271
Enterotoxin D (*sed*)	319
Enterotoxin E (*see*)	178
TSST-1 (*tst*)	445
Exfoliative toxin A (*eta*)	741
Exfoliative toxin B (*eta*)	629

PCR assay because the size of the amplimer was similar to that of the gamma amplimer. At the time of this study there was no reported method to amplify these genes in a multiplex PCR. The primers HLA-1 and HLA-2 for the alpha toxin gene and HLG-1 and HLG-2 for the gamma toxin gene were designed in this study. The primers HLD-1 and HLD-2 used for the delta toxin gene were as described previously by Janzon *et al.* (1989) (**Table 2.11**). The conditions for this multiplex PCR were developed in the present study.

According to this multiplex PCR method, of the 86 avian *S. aureus* isolates, 74 (87%) showed single bands corresponding to α-(~0.9 kb), γ-(~1.8 kb) and δ-(~0.5 kb) toxin genes. Twelve isolates gave no amplimers for γ-toxin but all of these gave bands for α and δ-toxin genes. These isolates were 31039, NFR, PD458, 217, 235, 82, 83, 86, 98, 496, 525 and 562 (**Table 3.23** and e.g. see **Figure 3.24**). Of the three avian coagulase-negative isolates tested, none of them showed reactions for α, γ and δ-toxin genes. Of the 13 human *S. aureus* examined by this method, eight isolates gave bands for α, γ and δ-toxins genes. These isolates were HUM-2, CN4575, 8530, HUM-G4, HUM-G6, HUM-271 and HUM-BB. Five isolates HUM-3, HUM-11, RN4220, HUM-294 and HUM-G3 gave bands for α and δ-toxins genes but these isolates did not give bands for the γ-toxin gene (**Table 3.23** and **Figure 3.24**). Of the four animal *S. aureus* isolates tested by this method, only isolate HOR-31912 gave bands for α, γ and δ-toxin genes. Isolates BOV-32276, BOV-66 and ZM-7 gave bands only for α and δ-toxins (**Table 3.23**). Human *S. epidermidis* isolate 134 did not show bands for α, γ and δ-toxin genes.

3.7.1.3 PCR for β-toxin gene

Primers HLB-1 and HLB-2 (**Table 2.11**) were designed in this study and the PCR conditions optimised. According to the design of the primers, the expected size of the gene fragment amplimer was ~1.8 kb.

Table 3.23 Detection of α, β, γ and δ toxin genes of *S. aureus* by PCR

Gene detected	Isolate*	No. of isolates
α, γ and δ	2A, 2B, 3A, 4, 5, 6, 7A, 7B, 8, 31174, 31175, 31176, 31177, 31178, 31179, 31180, 31181, 31182, 31183, 31036, 32528, E, K, SV6, SV289, 199, 214, PD486, PD380, PD27, 24, 34, 35, 36, 38, 77, 85, 99, 100, 104, 105, 122, 123, 562, 564, 572, 580, 582, 595, 596, 597, 598, 3, 11, 20, 25, 30, 48, 51, 52, 53, 88, 170, 172, 259, 329, 330, 352, 373, 390, 410, 451, 31, 33, 47, [a]HUM-2, [a]CN4575, [a]8530, [a]HUM-G4, [a]HUM-G5, [a]HUM-G6, [a]HUM-271, [a]HUM-BB, [b]HOR-31912	84
α and δ	31039, NFR, PD458, 217, 235, 82, 83, 86, 98, 496, 525, 562, [a]HUM-3, [a]HUM-11, [a]RN4220, [a]HUM-G3, [a]HUM-294, [c]BOV-32276, [c]BOV-66, [d]ZM-7	20
β (1.8 kb)	31178, 32528, 496, 525, [a]HUM-G1, [a]HUM-G4, [c]BOV-32276	7
β (1.2 kb) 7	[a]HUM-G2, [a]HUM-G3, [a]HUM-G5, [a]HUM-BB, [a]RN4220, [b]HOR-31912, [c]BOV-66	

*All are avian isolates except:
[a]: Human isolate
[b]: Horse isolate
[c]: Bovine isolate
[d]: Zoo mouse isolate

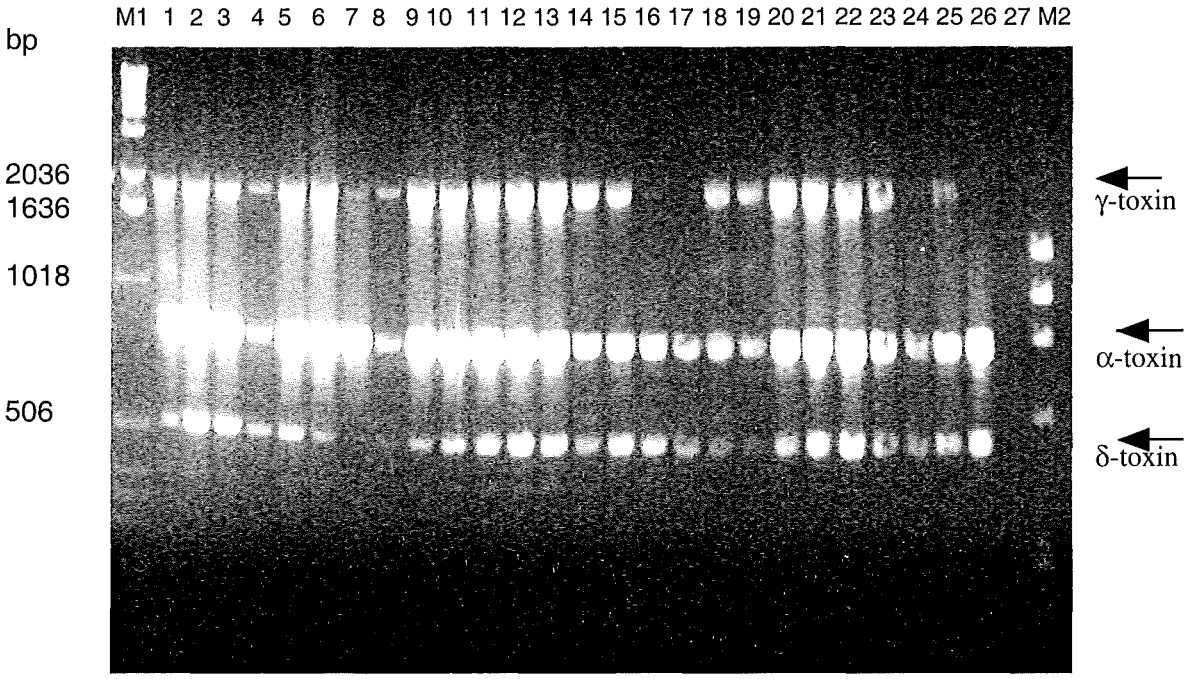


Figure 3.24 Examples of multiplex PCR for α, γ and δ-toxin genes in *S. aureus* isolates. Lanes M1 and M2 : 1 Kb DNA Lader and ϕX174 RF DNA/Hae III Fragments DNA Ladder respectively. Lanes 1-24: avian *S. aureus* isolates 4, 6, 8, 31175, 31176, 31179, 31180, 31181, 31182, 31183, 31036, 31039, PD458, PD380, 199, 217, 235, 53, 30, 170, 11, 31, 47 and 525, respectively. Lanes 25-26: human *S. aureus* isolates HUM-271 and HUM-294, respectively. Lane 27: avian *S. cohnii* isolate 31037 (see **Table 3.23**).

The results showed that only four of the 86 avian *S. aureus* isolates (5%) gave a single band (~1.8 kb) for the β-toxin gene. These isolates were 31178, 32528, 496 and 525 (**Table 3.23** and e.g. see **Figure 3.25**). Of the two avian coagulase-negative isolates examined by this method, neither showed any band. Of the twelve human *S. aureus* isolates tested, seven isolates (58%) showed a single band but with two different sizes (~1.8 kb and ~1.2 kb). The size of the band for isolates HUM-G1 and HUM-G4 was 1.8 kb but the size of the band for isolates HUM-G2, HUM-G3, HUM-G5, HUM-BB and RN4220 was 1.2 kb. Isolates HUM-2, HUM-3, CN4575, HUM-11 and 8530 did not produce a β-toxin band (**Table 3.23**). Human *S. epidermidis* isolate 134 did not show any band. Of the three animal *S. aureus* isolates examined, all of them had the β-toxin band. The size for isolate BOV-32276 was 1.8 kb but the size for isolates HOR-31912 and BOV-66 was 1.2 kb (**Table 3.23**).

3.7.2 Biological methods

For confirmation of the results of the molecular methods reported above, some of the positive and negative isolates were further investigated by biological assays for the toxins.

3.7.2.1 Enterotoxin A-D

Results from the commercially available test using the SET-RPLA TD900 kit (Oxoid) for the detection of staphylococcal enterotoxins A, B, C and D agreed completely with the findings of the multiplex PCR method for detection of the toxin genes. Avian *S. aureus* strains 31178 and 32528 were positive for enterotoxin B and A respectively by both methods and strains 2A, 2B, 3A, 4, 5, 6, 7A, 7B, 8, 31174, 31177 and 31181 were negative for all enterotoxins by both methods.

3.7.2.2 α-toxin

The production of staphylococcal α-toxin can be demonstrated by zones of clearing (haemolysin) around the colonies growing on agar containing 5% rabbit

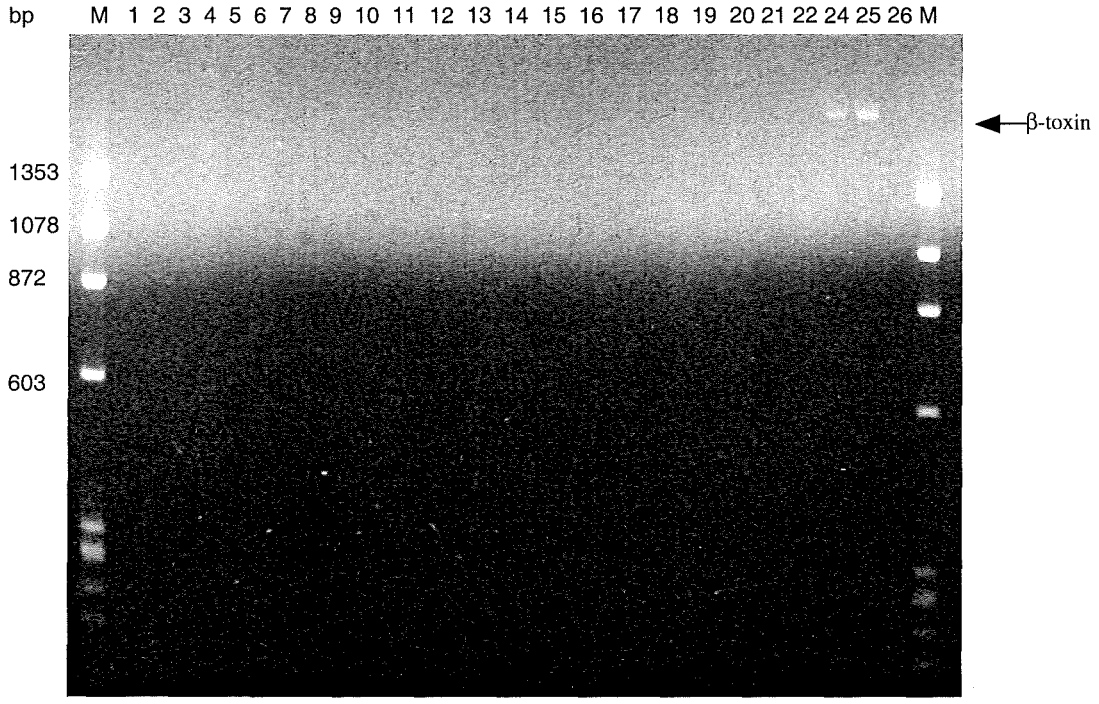


Figure 3.25 Examples of PCR amplification of β-toxin gene for *S. aureus* isolates. Lane M: ϕX174 RF DNA/Hae III Fragments DNA Ladder. Lanes 1-24: avian *S. aureus* isolates 4, 6, 8, 31175, 31176, 31179, 31180, 31181, 31182, 31183, 31036, 31039, PD458, PD380, 199, 217, 235, 53, 170, 30, 11, 31, 47 and 525, respectively. Lanes 25-26: human *S. aureus* isolates HUM-G2 and HUM-G3, respectively (see **Table 3.23**).

blood. There was good correlation between this method and the PCR method for detecting the α-toxin gene for all 86 avian, twelve human and six animal *S. aureus* isolates except that two avian *S. aureus* isolates 82 and 83 did not give a positive result with the biological method but they gave a positive result with the molecular method.

3.7.2.3 β-toxin

The production of staphylococcal β-toxin can be demonstrated by zones of clearing (haemolysin) around the colonies growing agar on containing 5% sheep blood. Again there was good correlation between this method and the PCR method for detecting the β-toxin gene for all 86 avian, ten human and five animal *S. aureus* isolates except that two avian *S. aureus* isolates 31039 and 562 did not give a positive result with the molecular method but they gave a positive result with the biological method.

Results for detection of toxin and haemolysin genes in the chicken isolates of *S. aureus* from N. Ireland and from the other, atypical avian strains not from N. Ireland are included in summary **Tables 3.20** and **3.21**, respectively.

3.8 GROWTH OF *S. aureus* STRAINS AT HIGH TEMPERATURES

The rationale for this experiment is that the body temperature of birds is 42 °C and that of humans is 37 °C. *S. aureus* strains adapted to birds may be able to tolerate higher growth temperatures. According to the literature (Kloos, 1998), the temperature growth range of *S. aureus* is 18-40 °C.

The results in **Table 3.24** at 44-45 °C indicates a difference between typical avian and some human and atypical avian strains. According to these results all typical avian and some of the atypical strains had heavy growth (+++) at 44-45 °C, but some of atypical and human strains had low growth (±).

The results in **Table 3.25** at 46-47 °C showed that only slight growth was observed with typical avian isolates 52, 53, 85, 105, 123, 170 and 329 and atypical strains 83, 562, 214, 217 and 32528. None of the human strains nor typical avian strains 24, 99, 100, 122 and 330 nor atypical avian strains 82, 86, 496, 525, 199, 235 and 31178 showed any growth at this temperature. However, when broth cultures from 46-47 °C were transferred to 37 °C and incubated overnight, all became turbid, and colonies were formed from all strains when the broth cultures from overnight at 46-47 °C were plated onto nutrient agar.

It appears that 46-47 °C is the growth limiting temperature for all strains but there was an indication from these results that some avian (typical and atypical) may be able to grow at slightly higher temperatures than human strains. It is interesting to note that none of the cultures were killed completely at 46-47 °C overnight and survivors were able to grow.

3.9 SEQUENCE ANALYSIS

For further analysis of the differences between avian and other strains of *S. aureus*, the complete sequence of a number of selected genes was determined, for typical and atypical avian strains and for some control strains.

Regions of the 16S rRNA (section 2.6.9.4), coagulase, *agrBDC* and cysteine protease consensus sequences of some of isolates in **Table 2.13** were compared to other sequences in the GenBank using the BLASTn search engine. Such analyses revealed that some of the sequences were novel but were similar to published sequences for other strains of staphylococci.

3.9.1 Sequence analysis of 16S rRNA gene

Regions of the 16S rRNA genes were amplified with primers C70 and C72 (**Table 2.11**) as previously described by Davies *et al.* (1996) for comparison of

Table 3.24 Growth of *S. aureus* strains at 44-45 °C

Type	Isolates	*Growth result
Typical avian	24, 52, 53, 85, 99, 100, 105, 122, 123, 170, 329, 330	+++
Atypical avian	82, 83, 86, 496, 525, 562, 199, 214, 235, 31178	+++
Atypical avian	217, 32528	±
Human	RN4220, 8530, HUM-G3, HUM-G4, HUM-G5, HUM-G6, HUM-BB	+++
Human	CN4575, HUM-G1, HUM-G2, HUM-11	±

*+++: Heavy growth, ±: Low growth

Table 3.25 Growth of *S. aureus* strains at 46-47 °C

Type	Isolates	*Growth result
Typical avian	52, 53, 85, 105, 123, 170, 329	±
Typical avian	24, 99, 100, 122, 330	-
Atypical avian	83, 562, 214, 217, 32528	±
Atypical avian	82, 86, 496, 525, 199, 235, 31178	-
Human	RN4220, 8530, HUM-G1, HUM-G2, HUM-G3, HUM-11 HUM-G4, HUM-G5, HUM-G6, HUM-11, HUM-BB, CN4575	-

*±: Low growth, -: No growth

the 16S rRNA gene sequences of isolates of *Pasteurella* (*Mannheimia*) *haemolytica* and *Pasteurella trehalosi.*

A 1439 bp region from the 16S rRNA gene of selected *S. aureus* strains was sequenced. When the sequences of avian *S. aureus* strains 24, 85, 99, 34, 98, 82, 496 and human *S. aureus* isolate RN4220 were compared, all were 100% identical and also identical to that of another human *S. aureus* strain ATCC 12600 published in GenBank (accession number L37597). The sequence of the 16S rRNA gene of bovine *P. haemolytica* PH2 was also determined, as a positive control, and the expected sequence was obtained (Davies *et al.,* 1996). The PCR products of this region of the 16S rRNA gene of the *S. aureus* strains examined were also indistinguishable (e.g. see **Figure 3.26**).

3.9.2 Sequence analysis of coagulase gene

A region of approximate 759 bp from PCR product of the coagulase gene of avian *S. aureus* strains 24 and 85 was also sequenced. When the sequences of typical avian strains 24 and 85 were compared (**Appendix III-b**), they were 100% identical but they were 97% identical to that of a human *S. aureus* isolate 213 (accession number X16457 published in GenBank) (not shown).

Atypical avian *S. aureus* strain 82 and human *S. aureus* strain RN4220 gave smaller PCR amplimers for the coagulase gene, of 684 bp which was sequenced (**Appendix III-b**). The sequence for avian strain 82 was 94% identical to that of human *S. aureus* isolate 213 listed in GenBank (accession number X16457) (not shown). The sequence for human isolate RN4220 (**Appendix III-b**) was 94% identical to that of the human *S. aureus* isolate BB sequence (GenBank accession number D00184; not shown).

Atypical avian *S. aureus* strains 496 yield a 685 bp sequence from the coagulase gene (**Appendix III-b**). When the sequence of this strain was compared with

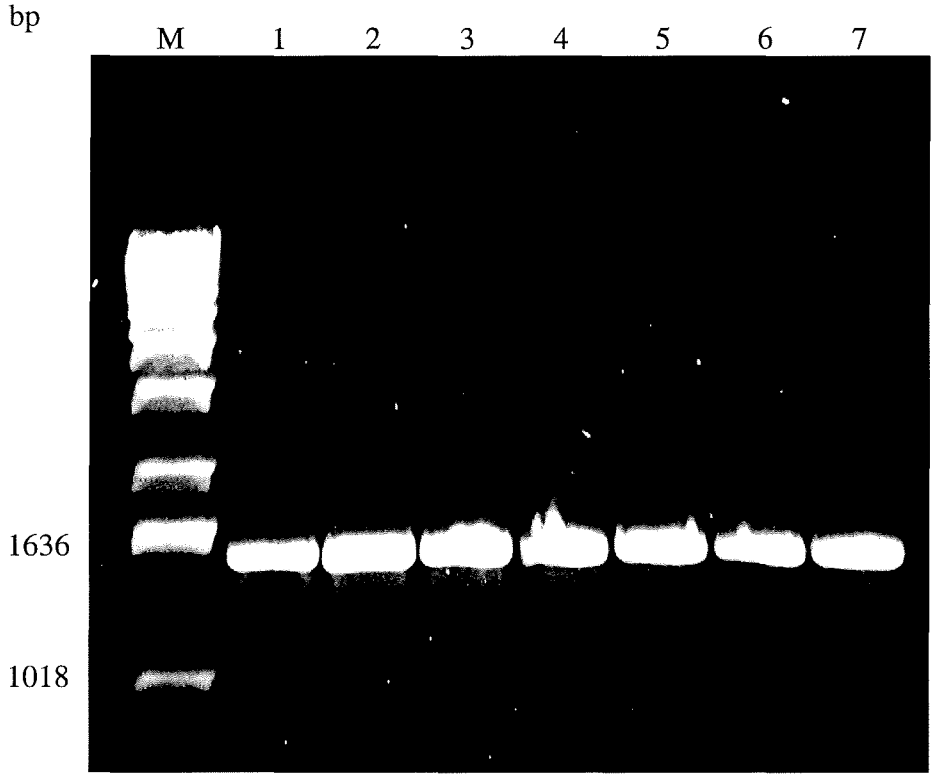


Figure 3.26 PCR of 16S rRNA gene for *S. aureus* isolates.
Lane M: 1 Kb DNA Ladder. Lanes 1-5: avian *S. aureus* isolates 24, 85, 99, 34 and 82, respectively. Lanes 6-7: human *S. aureus* isolates CN4575 and RN4220, respectively.

GenBank, it was 95% identical to the human *S. aureus* isolate 213 sequence with accession number X16457 published in GenBank.

The coagulase gene region of the other avian *S. aureus* strains (**Table 2.13**) was also sequenced. Sequence data for strains 99, 2B, 31174, PD27, 34 and 38 was 100% identical to that of strains 24 and 85. Strains 83, 86, 562, 217 and 235 were 100% identical to isolate 82. Also isolate 525 was 100% identical to strain 496.

3.9.3 Sequence analysis of *agrBDC* region

A region of 1005 bp from PCR product of the *agrBDC* region (section 3.6.1) of selected avian *S. aureus* strains 24 and 85 was sequenced (**Appendix III-c**). When the sequences of these isolates were compared, they were 100% identical but were 98% identical to that of human *S. aureus* isolate SA502A sequence published in GenBank (accession number AF001782; not shown). Analysis of the 144 bp sequence of the *agrD* region of 24 and 85 isolates (**Appendix III-d**) showed that they belonged to group II of the autoinducing peptides [AIPs] processed from a propeptide encoded by *agrD* (see section 1.3).

A region of 983 bp from PCR product of the *agrBDC* region of selected avian *S. aureus* strain 82 was sequenced (**Appendix III-c**). When the sequence of this isolate was compared in GenBank, it was 98% identical to that of human *S. aureus* isolate RN4282 (accession number U85097; not shown). Analysis of the 141 bp sequence of the *agrD* of this isolate (**Appendix III-d**) showed that it belonged to group I of the AIPs.

A region of 992 bp from PCR product of the *agrBDC* region of human *S. aureus* strain RN4220 was sequenced (**Appendix III-c**). When the sequence of this isolate was compared, in GenBank it was 99% identical to that of human *S. aureus* RN4282 (accession number U85097; not shown). Analysis of the 141 bp sequence of the *agrD* of this isolate (**Appendix III-d**) showed that it belonged to group I of the AIPs.

A region of 1005 bp from PCR product of the *agrBDC* region of selected avian *S. aureus* strain 496 was sequenced (**Appendices III-c**). When the sequence of this isolate was compared in GenBank, it was 99% identical to that of human *S. aureus* isolate RN8462 (accession number AF001783; not shown). Analysis of 144 bp sequence of the *agrD* of this isolate (**Appendices III-d**) showed that it belonged to group III of the AIPs.

The *agrBDC* region of the other avian *S. aureus* strains (**Table 2.13**) was also sequenced. Sequence data for strains 99, 2B, 31174, PD27, 34, 38, 104, 595, 35 and 98 was 100% identical to that of strains 24 and 85. Strains 83, 86, 562, 217 and 235 were 100% identical to isolates 82 and RN4220. Also isolates 525, 31178 and 32528 were 100% identical to strain 496.

According to these results, typical avian isolates are in group II, but atypical avian and human isolates are in groups I and III, of the autoinducing peptides [AIPs] processed from a propeptide encoded by *agrD* (see section 1.4).

4. DISCUSSION

4.1 THE ISOLATES

There was no type strain of avian *S. aureus* available in the major culture collections but the strain selected as a control, *S. aureus* strain 24, was obtained from a case of proximal femoral degeneration in a broiler from Northern Ireland. This strain was used as a standard strain in a study by McCullagh *et al.* (1998) in that the majority (85%) of isolates from diseased chickens in commercial flocks and 71% of the hatchery isolates had a PFGE profile indistinguishable from that of strain 24 and were therefore classified as "type 24". The sources of the isolates in the present study were Scotland, England, N. Ireland, America and Sri Lanka. More than half of the isolates were from N. Ireland. The isolates were mainly from clinically-diseased birds (**Table 2.1**). Other avian, human and animal coagulase-positive and coagulase-negative species served as control organisms in the various test procedures.

One of the main aims of this project was to devise a simple, rapid and reliable typing method for avian isolates of *S. aureus*.

Although phage-typing has been used to type *S. aureus* from poultry (Shimizu, 1978; Thompson *et al.,* 1980a; Kibenge *et al.,* 1982a; Shimizu *et al.,* 1986), the poultry phage set has not been widely employed in recent years. One of the problems in applying a phage typing system on a national or international basis is its effectiveness in lysing strains from different geographic regions (Shimizu, 1979). Another problem is that it is not a static system, as it must respond to the emergence of new strains of staphylococci that are nontypable by the latest set of phages (Kloos and Lame, 1991). Also, in some instances, the observation that phage typing systems produce illogical or inconsistent results may indicate problems in methodology or interpretation (Kristjánsson *et al.,* 1994).

Biotyping in many clinical microbiology laboratories is now routinely and reliably performed using automated or standardised systems designed for species identification. However, attempts to use biochemical reactions for strain

differentiation of *S. aureus* and of coagulase-negative staphylococci have proved largely unsatisfactory both because of poor reproducibility among independent isolates representing the same strain and relatively weak discriminatory power (Mickelsen *et al.,* 1985; Tenover *et al.,* 1994).

One of the most discriminatory methods for typing bacterial isolates in current use is PFGE analysis of genomic DNA that has been restricted with infrequent cutting enzymes. This techniques has been used successfully with avian *S. aureus* (Saulnier *et al.,* 1993; Tenover *et al.,* 1994; McCullagh *et al.,* 1998). For example, McCullagh *et al.* (1998) used the PFGE method for typing avian isolates of *S. aureus* in N. Ireland. The bacterial grouping was based on the number of similar bands present between restriction fragment patterns. The groups consisted of strains (a) identical to type strain 24, showing no band differences, (b) closely related, showing 1-3 band differences, (c) possibly related, showing 4-6 band differences and (d) unrelated, showing more than 6 band differences. McCullagh *et al.* (1998) used PFGE analysis of *SmaI* restriction patterns to type 109 isolates of *S. aureus* collected from broiler farms and hatcheries in Northern Ireland. Forty-seven isolates from clinical conditions in broilers and 62 strains from hatcheries, were examined. The PFGE patterns demonstrated a similarity between 85% of strains from clinical sources and 71% of the hatchery isolates. The association of disease with the predominant strain type (type 24) and the presence of these same strains in the hatchery indicated that the hatchery was a potential source of the infection for clinical broiler disease. Comparison of a number of isolates from the same broiler houses demonstrated the prevalence of one PFGE type per house, and was in agreement with the findings of a study using phage typing (Kibenge *et al.,* 1982a). Although the mechanism of spread of *S. aureus* infection through poultry flocks is not understood, Thompson *et al.* (1980b) had earlier suggested that the hatchery played an important role in the spread of infection to rearing farms and has a role in the dissemination of *S. aureus* in poultry. The eggs supplied from the broiler parent farm have been recognised as a potential source of salmonella

infection in the hatchery (Davies and Wary, 1994), and could play a similar role in the spread of *S. aureus* infection associated with clinical disease in broilers.

For the present study, a large number of avian isolates that had been biotyped and typed by PFGE were kindly provided by Dr McCullagh. However PFGE typing is technically demanding and time consuming (Maslow *et al.,* 1993; Birren and Lai 1993; Cookson *et al.,* 1996). The intention was to see if a simple rapid typing method could be derived that was as discriminatory or more discriminatory than PFGE. A collection of 51 PFGE-typed isolates were used, including a large number of type 24 isolates.

It is well known that *S. aureus* strains produce various extracellular active substances, such as coagulase, haemolysins, nuclease, acid phosphatase, lipase, proteases, fibrinolysin and various toxins. These active substances are thought to contribute to the pathogenicity of the organism and the majority of them have been purified and their biological properties extensively examined by many researchers (reviewed by Dinges *et al.,* 2000). The aim of this project was not only to investigate typing methods for avian strains but also to characterise some of the virulence properties. By PCR amplification using primers for known virulence-associated genes, it was hoped to gain insight into both of these aspects.

4.1.1 Biochemical properties of the isolates

Conventional biochemical properties. The main purpose of determination of the biochemical activities of these isolates was to identify or, for most of them, to confirm their identity, before use in other experiments. These tests were chosen on the basis of previous work where they were reported to give clear positive or negative results for the species (Kloos and Lambe, 1991; Kloos, 1998). With the biochemical tests used, results for coagulase-positive isolates were very similar to those for coagulase-negative isolates, in agreement with

previous reports (Kloos and Schleifer, 1975a,b; Kloos and Lambe, 1991; Kloos, 1998).

The initial identification of the staphylococci were made on the basis of culture on mannitol salt agar (this medium inhibits the growth of Gram-negative bacteria, but allows the growth of staphylococci and certain other Gram-positive bacteria). Gram stain and production of catalase were also used for preliminary identification of staphylococcal species. Only two biochemical tests, namely coagulase (human and rabbit plasma) and DNase plate culture test, were able to differentiate coagulase-positive and coagulase-negative staphylococci (Bergey's Manual, 1974). All avian *S. aureus* isolates in this study gave positive results for these tests, as reported previously for avian isolates (Hájek and Maršálek, 1976; Devriese and van de Kerckhove, 1979).

However, biochemical properties have been used to differentiate isolates of coagulase-positive *S. aureus* of human, animal and avian origin. There were four different biotypes identified among the *S. aureus* isolates and one biotype for *S. intermedius* in a study by Hájek and Maršálek (1976). Gibbs *et al.* (1978a) showed three biotypes for isolates of *S. aureus*. Devriese (1984) and Isigidi *et al.* (1990) showed five biotypes for *S. aureus*, whereas Harvey *et al.* (1982) showed two biotypes for *S. aureus*. These observations of biotyping groups were based on panels of 6-9 different tests. In general, the biochemical methods are labour intensive and time consuming and poorly discriminatory.

Usefulness of Rapidec Staph and API Staph. To expedite the process of identifying staphylococcal species and subspecies in the clinical laboratory, several manufacturers have developed rapid identification kits and automated systems requiring only a few hours to one day for completing the tests. With these products, identification of most species and subspecies can be made with an accuracy of 70->90% (Gemmell and Dawson, 1982; Bannerman *et al.,* 1993).

The Rapidec Staph system has been used for the identification of the main staphylococci species *S. aureus*, *S. epidermidis*, *S. saprophyticus*, but it is not able to differentiate other staphylococcus species. The Rapidec Staph system was used in this study as a supplementary method for the confirmation and identification of the isolates. The results obtained for the different species were very similar to the findings of Kloos (1998). In general, all *S. aureus* isolates in the present study gave identical profiles in this panel of four tests. There were no differences between typical avian isolates and human or animal strains.

API Staph is an identification system for the genera *Staphylococcus* and *Micrococcus*, using standardised and miniaturised biochemical tests with a specially adapted database. In this study, the API Staph system was used primarily for confirmation and identification of the *S. aureus* isolates and other species. The results obtained by API Staph system for the species were done according to the manufacturer's (bioMérieux) instructions and were based on the classification of Kloos and Schleifer (1975a, b), and Kloos and Lambe (1991). In general, all *S. aureus* isolates in the present study gave identical profiles in this panel of 20 tests. These were no differences between typical avian isolates and human or animal strains. Also, this system showed that all avian isolates, like human strains, produced urease. The only previous report of urease activity in avian *S. aureus* isolates was in a comparison of strains from freshly slaughtered poultry and strains "endemic" to processing plants (Mead *et al.*, 1989). It was reported that a higher proportion of "endemic" strains produced urease. These results therefore differ from the present results where the avian isolates were obtained from live birds and all produced urease.

Rapidec Staph and API Staph systems are useful for identification of *Staphylococcus* species but Watts and Yancey (1994) have reported that commercial biochemical kits available for the determination of coagulase-negative staphylococcal species are unreliable for the identification of veterinary pathogens, because they were developed for human isolates.

Rapid identification of *S. aureus* by PCR: The rapid identification of *S. aureus* is important so that the appropriate antibiotic therapy can be initiated, and this bacterial species must be differentiated from coagulase-negative staphylococci. Several methods for rapid identification of *S. aureus* have been evaluated. Some of these methods include coagulase tests (Davis *et al.,* 1992), a panel of commercial agglutination tests (Wilkerson *et al.,* 1997), a hybridization test for rRNA (Davis and Fuller, 1991) and an enzymatic test for the detection of the thermostable nuclease (Madison and Baselski, 1983). Although identification with the thermonuclease enzyme test has shown an excellent correlation with the conventional identification of *S. aureus* isolates (Madison and Baselski, 1983), false-positive results due to thermonuclease activity in some strains of coagulase-negative staphylococci may occur (Chesneau *et al.,* 1993; Kloos and Bannerman, 1994). In addition, some streptococcal isolates may give a positive thermonuclease test (Park *et al.,* 1980). The hybridization test for rRNA showed excellent specificity for *S. aureus* but demonstrated an evident lack of sensitivity for detection from blood cultures (Davis and Fuller, 1991).

Brakstad *et al.* (1992) have developed a PCR assay specific for *S. aureus* by targeting the *nuc* gene coding for staphylococcal thermonuclease. This assay was shown to be suitable for diagnostic purposes and this method was used in the present study for identification *S. aureus* isolates. Another simple and rapid PCR assay that Martineu *et al.* (1998) developed for the diagnosis of *S. aureus* infections from blood culture or clinical specimens was also used in the present study. These two rapid PCRs were applied to all *S. aureus* avian, human and animal isolates in this study and the results of the two methods gave complete agreement with each other and with the other biochemical and conventional methods. All isolates identified as *S. aureus* gave positive reactions with identical bands at 300 bp and 108 bp for the nuclease region and the species-specific chromosomal DNA fragment of *S. aureus,* respectively. None of the avian, human or animal coagulase-negative or *S. intermedius* isolates showed

any amplified bands by these methods. These rapid and specific tests for *S. aureus* are therefore a good alternative to biochemical typing procedures.

4.2 PLASMID PROFILE PATTERNS

Plasmid profile analysis is a molecular fingerprinting method that has been used for discrimination between *S. aureus* isolates (Dodd *et al.,* 1987; 1988; Gillespie *et al.,* 1990; Jovanovicacute *et al.,* 1991; Matthews *et al.,* 1992; Montserrat *et al.,* 1994; Tenover *et al.,* 1994; Aarestrup *et al.,* 1995b; Damiani *et al.,* 1996; Yoshida *et al.,* 1997; Lange *et al.,* 1999).

In a study of *S. aureus* isolates from different poultry processing plants (Dodd *et al.,* 1987; 1988), plasmids were detected in 72% of strains but the proportion of plasmidless strains isolated varied amongst plants. Plasmids were detected in 76% of strains from plant 1; 96% of strains from plant 2; but only 46% of strains from plant 3 (Dodd *et al.,* 1987). Also Dodd *et al.* (1987; 1988), showed that plasmids profiles could be used to distinguish endemic strains of *S. aureus* present in processing plants from those of the skin flora of turkeys and chickens. Earlier, differences in plasmid content were found between strains of *S. aureus* associated with live poultry (Witte *et al.,* 1977; Thompson *et al.,* 1980b) and those isolated from processed poultry which were termed endemic strains. However, as it has been shown that strains other than endemic types may be associated with processed poultry carcasses (Adams and Mead, 1983), it is not clear whether these "endemic" strains were those truly indigenous to the plants or were part of the original skin flora. Thompson and Holding (1986) showed that plasmids were more common in strains of *S. aureus* characteristically associated with live poultry than with strains endemic in poultry plants and strains of human origin. They also showed that a plasmid of size 1.65 kb was present in all seven isolates belonging to the poultry biotype.

In the present study, plasmids of 1.6, 4.4 and 16 kb were common in most of the avian *S. aureus* (**Table 3.4**) from different sources.

A high proportion (95%) of avian *S. aureus* isolates had plasmids and the profiles of most of the isolates were similar. Their sizes ranged from 1.6 to 16 kb. All the avian N. Ireland *S. aureus* isolates related to PFGE type 24, except isolate 77, and isolates 34 and 38 that were closely related to PFGE type 24 according to the designation of McCullagh *et al.* (1998) had similar plasmid profiles. The other N. Ireland isolates that were possibly related or unrelated to type 24 according to the McCullagh *et al.* (1998) designation had different plasmid profiles (**Table 3.4**).

The present study has also demonstrated a close correlation between tetracycline resistance and the presence of a 4.4 kb plasmid. A plasmid of this size was present in all avian *S. aureus* isolates, from different geographic regions, examined, that were resistant to tetracycline except isolate E. This isolate from a Goshawk had two other plasmids of 1.6 and 16 kb, and plasmids of these sizes were common in other isolates but they did not appear to encode resistance to tetracycline (**Table 3.4**).

The present study has demonstrated a close correlation between those isolates resistant to one or more of penicillin G, kanamycin, tobramycin, gentamicin, pefloxacin, erythromycin, lincomycin and nitrofurantoin and the presence of a plasmid of approximate 16 kb (**Tables 3.4** and **3.9**). Of the strains resistant to any of these antibiotics, only strain 32528 did not have the 16 kb plasmid but was resistant to penicillin G. It may be that this large plasmid is commonly associated with antibiotic resistance genes, but this needs further investigation. It is interesting to note that the only plasmid common to the human strains used in this study also had a mol.wt of 16 kb. Laufs *et al.* (1978) and Kloos *et al.* (1981) had previously presented data showing that *S. aureus* strains of human origin had plasmid profiles similar to those from poultry.

Coagulase-negative strains also possessed plasmids but, in general, the profiles were distinct from those of the coagulase-positive strains. The plasmid profiles results for avian coagulase-negative strains showed that only two isolates had a 3 kb plasmid which was the same size as a plasmid in some of the avian *S. aureus* strains. Further work would be required to determine if these plasmids are similar and transmissible between strains (**Tables 3.4** and **3.5**).

Some of the animal staphylococcal (coagulase-positive and negative) isolates possessed a plasmid of 16 kb, similar in size to that in some avian *S. aureus* (**Tables 3.7**, **3.8** and **3.4**). In general, however the plasmid profiles of the seven bovine strains examined did not resemble those from poultry strains. Baumgartner *et al.* (1984) studied plasmids in strains of *S. aureus* from mastitis cases and showed that 70% of 97 strains carried plasmids, but they did not record the sizes of the plasmids.

4.3 ANTIBIOTIC SENSITIVITY PATTERNS

Resistance typing may also be a useful primary typing tool for *S. aureus* isolates of unrelated geographical origins, as suggested by Struelens *et al.* (1992). Aarestrup *et al.* (1995b) have suggested that antibiogram typing and plasmid profiles might be helpful in solving epidemiological problems concerning strains, which are assigned to the same type by other typing techniques.

The antibiotic sensitivity patterns of the avian *S. aureus* isolates in the present study showed that 96% were resistant to most of the commonly used antibiotics (**Table 3.9**). According to the sensitivity patterns, 71% were resistant to tetracycline, kanamycin, tobramycin and gentamicin, fifty (67%) isolates were resistant to pefloxacin, fourteen (19%) were resistant to penicillin G, two (3%) were resistant to erythromycin, one (1%) was resistant to nitrofurantoin and one (1%) was resistant to lincomycin (**Table 3.9**). 85% of avian *S. aureus* isolates were resistant to more than one of these antibiotics. All Irish, American and Sri

Lankan avian *S. aureus* isolates in the present study were resistant to kanamycin, tobramycin and gentamicin.

Lukásová *et al.* (1986) showed that more than 50% of the *S. aureus* strains isolated from slaughtered poultry were resistant to penicillin, erythromycin, and streptomycin and less than 50% resistant to tetracycline. Papadopoulou *et al.* (1997) showed that strains of *S. aureus* from eggs were resistant to penicillin G, tetracycline, erythromycin, clindamycin, cefalosporins, oxacillin, gentamicin, chloramphenicol and tobramycin. Aarestrup *et al.* (2000) reported that a high frequency (30%) of avian *S. aureus* isolates from Denmark was resistant to ciprofloxacin but only 7% of avian *S. aureus* isolates were penicillin resistant. Resistance to sulphamethoxazole was observed among 19% of avian *S. aureus* isolates. 24% of the avian *S. aureus* isolates were resistant to erythromycin and 79% of these isolates contained the *ermA* gene, whereas the remaining isolates contained the *ermC* gene. 47% of the avian *S. aureus* isolates were tetracycline resistant and all contained the *tet(K)* gene. A single avian *S. aureus* isolate also contained the *tet(M)* gene. In a study by Schwarz *et al.* (1998), tetracycline resistance was observed in 29.3% of staphylococcal isolates. The level of tetracycline resistant isolates varied with respect to the animal host: 75% from cats, 58.1% from mink, 41.4% from pigeons, 28% from guinea pigs, 26.1% from horses, 21.4% from ducks, 14.9% from rabbits and 11.8% from turkeys. Multi-resistant isolates were more frequently isolated from horses, rabbits, mink and pigeons, mainly involving additional resistances to ampicillin, chloramphenicol or erythromycin. In contrast, multi-resistance was seldom detected in tetracycline resistant isolates from cats, turkeys, ducks or guinea pigs. The antibiotic resistance patterns depended on the staphylococcal species and on the animal host. Moreover, the determination of antibiotic resistance patterns might provide important information towards specific control measures.

Animal husbandry is an important user of antibiotics for growth promotion in a range of animals including poultry, cattle, salmon and catfish (Levy, 1992).

Taking into consideration also that antibiotics such as ampicillin, erythromycin, tetracycline, gentamicin and chloramphenicol have been used for the control of infections by large poultry breeders, and comparing the resistance of the isolated bacterial strains to these specific antibiotics, it has been suggested that resistant strains could be passed to humans though the food chain (Papadopoulou *et al.*, 1997). Schwarz *et al.* (1998) suggested that there was a correlation between the number of tetracycline resistant isolates in the staphylococcal population and the antibiotic usage in the respective animal host. Resistant isolates from animals which suffered from clinical staphylococcal infections such as canine pyoderma had most probably been in contact with antibiotics prior to sampling. Moreover, most resistant isolates from mink were from animals which had definitely received tetracyclines and/or other antibiotics. Carrier pigeons from which resistant staphylococcal isolates were obtained had also received antibiotics for prophylactic purposes prior to sampling. On the other hand, in turkeys and ducks raised without any antibiotic treatment there were only low levels of tetracycline-resistant staphylococci. The observation that no mupirocin resistant isolates were detected strongly reflected the restriction of this antibiotic to exclusive use in human medicine.

The present study showed that 71% of the avian *S. aureus* and 70% of the strains from commercial flocks were resistant to tetracycline, suggesting that this antibiotic is widely used in commercial poultry rearing. As described in the previous section, there was a strong correlation between tetracycline resistance and the presence of a 4.4 kb plasmid. Purification of this plasmid and transfer by electroporation to a tetracycline sensitive strain conferred tetracycline resistance on the recipient and proved that the plasmid encoded tetracycline resistance. This finding indicated that the 4.4 kb plasmid found in many strains is correlated with tetracycline resistance (**Tables 3.4** and **3.9**). In a study by Needham *et al.* (1994) of plasmids from human *S. aureus* that mediate resistance to mupirocin and tetracycline, five isolates showed a plasmid of 4.4 kb, two isolates had plasmids of 4.4 kb and 20 kb and one isolate had a 5.2 kb plasmid together with

a plasmid of about 20 kb. There are no previous published reports mentioning a plasmid of 4.4 kb conferring tetracycline resistant in avian strains of *S. aureus*.

The presence of high levels of antibiotic resistance in avian *S. aureus* is worrying. It may be that such strains could be passed to humans via the food chain as suggested above although the pathogenic potential of avian strains for man is unknown. Alternately, it may be that antibiotic resistant genes and antibiotic resistant plasmids can develop in avian strains and then be transmitted horizontally to human strains.

4.4 FINGERPRINTING OF ISOLATES BY PCR METHODS

In the present study PCR typing methods were investigated for their suitability for avian strains because of their successful application to typing *S. aureus* from human cases. A number of authors have proposed that PCR typing methods are a suitable replacement for conventional phage typing methods for human *S. aureus* strains (Saulnier *et al.,* 1993; Tenover *et al.,* 1994; Bamnerman, *et al.,* 1995; van Belkum, *et al.,* 1995; Saruta *et al.,* 1997; Forsman *et al.,* 1997; Deplano and Struelens, 1998; Deplano *et al.,* 2000; Enright *et al.,* 2000).

The ideal system for the typing of *S. aureus* strains should be easy, rapid, reliable, highly discriminatory, and reproducible. Furthermore, it should be suitable for widespread use, so that the genotyping results obtained in different laboratories or countries can be compared.

4.4.1 PCR-ribotyping

PCR amplification of the 16S-23S spacer regions with universal primers (method 1) showed that this method readily distinguishes most avian isolates from human isolates. The simple PCR assay presented in this study is useful for the immediate typing of *S. aureus*. All 86 avian *S. aureus* isolates gave fingerprints, with approximately nine bands ranging from ~0.4-~0.8 kb. On the

basis of nine distinct bands, twelve groups were recognised for the 86 avian *S. aureus* isolates and each was assigned a number (**Table 3.12**). These polymorphisms are presumably due to variations in the number of, and distance between, the neighbouring *rrn* genetic loci. The majority (73%) of avian *S. aureus* strains, associated with clinical infections in the different countries investigated in the present study, had a PCR-ribotyping pattern like that of isolate 24 (**Table 3.12**). The ability to type avian *S. aureus* isolates by this PCR method appeared to be similar to that of PFGE in that all PFGE type 24 strains had the same ribotype (type 1 by method 1). Strains unrelated to type 24 by PFGE had different ribotyping profiles (types 2, 3 and 5). However, this ribotyping method was somewhat less discriminatory than PFGE in that two strains "closely related" to type 24 by PFGE (distinguishable by PFGE) had the same ribotyping profile as the type 24 strains, whereas one closely related and one possibly related strain had the same ribotyping profile as the strains unrelated to type 24.

The results obtained in the present study demonstrated the potential of this typing method especially in combination with other methods in epidemiological investigations (see summary **Table 3.20**). Application of this method was easy and it has advantages in terms of speed and simplicity over the PFGE typing method. In addition, PCR-ribotyping method 1 could differentiate two of the avian strains, 496 and 525 which were classified as UR (unrelated to type 24 by PFGE) from the rest of the UR strains (**Table 3.20**). Another group of atypical avian strains, which had not been typed by PFGE, are listed in **Table 3.21**. These strains mainly showed profiles by PCR-ribotyping method 1 that were distinct from those of the other avian strains (**Table 3.12**).

The strains that reacted by ribotyping method 2 are discussed further in section 4.8. The majority of the more typical avian strains did not react by this method and the implication of this, for distinguishing between typical avian strains of *S. aureus* from other strains, are discussed in section 4.8. The reason for the lack of

reaction of typical avian strains by PCR-ribotyping method 2 may be sequence variation between avian and other strains in the primer regions. A comparison of the full sequence of this 16S-23S spacer region of avian strains with those of human and animal strains could reveal other differences between these strains. Twelve avian *S. aureus* strains resembled human and animal strains in giving a reaction with PCR-ribotyping method 2 (**Table 3.13**) (this is discussed further in section 4.8). This method investigates the level of sequence variation in the 16S-23S spacer regions between different isolates and has clear potential use as a rapid means of distinguishing typical avian *S. aureus* isolates from atypical avian and non-avian *S. aureus* isolates. The conventional microbiological and biochemical methods for identifying and biotyping isolates are time consuming, by comparison with this type of PCR method.

4.4.2 REP-PCR

REP sequences were first described in *E. coli* and *Salmonella typhimurium* but they are widespread in bacteria (Stern *et al.,* 1984; Hulton *et al.,* 1991; Versalovic *et al.,* 1991). The repetitive element sequence-based PCR (REP-PCR) used in the present study was described for the molecular genotyping of human *S. aureus* strains by DelVecchio *et al.* (1995). This high-stringency PCR fingerprinting method was based on a repetitive sequence found in *Mycoplasma pneumoniae* (Wenzel and Hermann, 1988) but it was found to generate strain-specific DNA fragments when *S. aureus* DNA was used as an amplification template. van der Zee *et al.* (1999) reported that analysis of a collection of human *Staphylococcus* strains, described earlier by Tenover *et al.* (1994) and van Belkum *et al.* (1995), showed that the REP-PCR typing method was as discriminatory as arbitrarily-primed PCR (AP-PCR) and PFGE. AP-PCR is a little faster to perform than REP-PCR, but the reproducibility of REP-PCR, which is highly important when used for epidemiological purposes, was found to be better than that of AP-PCR. If REP-PCR is compared to PFGE, the most important advantage of REP-PCR is its easy and fast performance. The reproducibility of REP-PCR is comparable to that of PFGE. Another PCR-based

typing method for *S. aureus* strains known as inter-IS256 PCR, which compares best to REP-PCR typing with regard to ease of performance and reproducibility, has been described by Deplano *et al.* (1997). On the other hand Deplano *et al.* (2000), suggested that it would be difficult to fully standardise these PCR typing methods using current technology, because of equipment variation between laboratories.

The present study was performed to assess the usefulness of REP-PCR for genotyping of avian *S. aureus* strains and to compare this with ribotyping and PFGE. Of the 86 avian *S. aureus* strains, seven groups (**A-G**) were recognised (**Table 3.14** and **Figure 3.14**). The patterns were complex, consisting of multiple bands ranging from 0.15-3 kb. All avian *S. aureus* isolates related to PFGE type 24, closely and possibly related to PFGE type 24 according McCullagh *et al.* (1998) were in group **A** and therefore REP typing was less discriminatory than PFGE. All of the avian *S. aureus* isolates unrelated to type 24 according McCullagh *et al.* (1998) showed different REP profiles. In comparison with other typing methods investigated in the present study, the discriminatory power of REP-PCR in genotyping avian *S. aureus* was less than that of PCR-ribotyping method 1 (12 types) (**Table 3.12**) and RFLP analysis of the coagulase gene (8 types) (**Table 3.16**) but greater than that of a polymorphic locus, e.g. the X-region of the protein A gene (*spa*) (five types) (**Table 3.18**) (see below).

Recent information from the *S. aureus* genome sequence (Kuroda *et al.,* 2001) has shown the presence of unique repetitive sequences. According to this recent information, primers could be designed for use specifically with *S. aureus* and a comparison made with REP-PCR.

4.4.3 PCR amplification and RFLP analysis of a region of the coagulase gene

Coagulase production is an important phenotypic determinant used for the identification of *S. aureus* isolates from human and animal infections but there

are conflicting data concerning the importance of coagulase as a major virulence factor. It is currently believed that coagulase may not be an important virulence factor because mutants defective in coagulase production created by site-directed allele replacement were not reduced in virulence in a particular model of *S. aureus* infection (Phonimmdaeng *et al.,* 1990). However, because of gene variability, coagulase genotyping has proven to be a simple and powerful epidemiological tool to discriminate between coagulase-positive human and bovine *S. aureus* strains (Goh *et al.,* 1992; Schwarzkopf *et al.,* 1994; Aarestrup *et al.,* 1995a; van Belkum *et al.,* 1997; Su *et al.,* 1999; Hookey *et al.,* 1999; Shopsin *et al.,* 2000).

Goh *et al.* (1992) reported that the coagulase gene exists in multiple allelic forms, in part because of the existence of variants within the 3′-end of the coding region. This contains a series of repeating 81-bp DNA sequences and differs both in the number of tandem repeats and the location of *AluI* restriction sites among different isolates. Goh *et al.* (1992) developed a novel typing method for human *S. aureus* based on PCR amplification of the variable region of the coagulase gene followed by *AluI* restriction enzyme digestion. There was excellent correlation of the RFLP patterns of different strains with typing by multi-locus enzyme electrophoresis at chromosomal loci. Su *et al.* (1999) investigated the coagulase gene polymorphism of *S. aureus* isolates obtained from bovine mastitic milk in order to determine the resistance of dominant and rare coagulase genotypes to the bactericidal activities of bovine blood neutrophils. A total of 453 isolates were collected from four countries: the Czech Republic, France, Korea and the United States. The isolates were subtyped into 40 types by RFLP analysis of the coagulase gene.

The present study on the coagulase region showed that there were different genotypes among *S. aureus* isolates from avian, human and animal sources. The 86 avian *S. aureus* isolates could be classified into four distinct groups based on their PCR-amplified coagulase gene products and eight distinct groups based on

their unique *AluI* RFLP profiles (**Tables 3.15** and **3.16**). The latter method was therefore more discriminating than the former, e.g. for isolates 31178, 35, 36, 98, 104, 595, 580, 597, 598, 496, 525, E and 32528 (**Tables 3.15** and **3.16**). Also, comparison of the coagulase gene RFLP with PFGE results on the N. Ireland isolates showed that avian *S. aureus* isolates of type 24 and closely-related type 24 isolates had identical band patterns. The possibly-related and unrelated strains could be grouped into 3 different types (**Table 3.16** and **Figure 3.16).** The unrelated isolates 82, 83, 86, 496, 525 and 562 had bands common to both avian and human strains (**Table 3.16** and **Figure 3.16**). Thus this method has limited value in differentiating typical avian strains but human and animal isolates were readily distinguishable from typical avian isolates after restriction. Coagulase gene typing gave the same discrimination as PCR-ribotyping method 1 (**Tables 3.13** and **3.16**).

Shopsin *et al.* (2000) has reported recently that coagulase gene (*coa*) short sequence repeat region sequencing could be used to measure relatedness among a collection of temporally and geographically diverse methicillin-resistant human *S. aureus* isolates. Their results showed that *coa* polymorphism is free of strong selective pressure and had a low index of variation that might be useful for long-term epidemiological investigation. Also Chiou *et al.* (2000) has compared PFGE and coagulase gene restriction profile (CRP) analysis techniques in the molecular typing of human *S. aureus* isolates. These techniques were used to analyse 71 isolates recovered from nine food-borne disease outbreaks. Twenty-two PFGE profiles and 11 CRPs were identified, with discrimination indices of 0.86 and 0.72, respectively.

4.4.4 PCR amplification and RFLP analysis of X-region of the protein A gene

The key biological property of protein A is its ability to bind to the Fc portion of IgG antibodies (Forsgren and Sjöquist, 1966). The expression of protein A (*spa*) is repressed by global regulatory loci *sarA* and *agr*. Although SarA may directly

bind to the *spa* promotor to downregulate *spa* expression, the mechanism by which *agr* represses *spa* expression is not clearly understood (Cheung *et al.,* 2001). The *spa* gene is composed of approximately 2150 bp and harbours a number of functionally distinct regions, an Fc-binding region, the so-called X-region, and, at the C-terminus, a sequence required for cell wall attachment (Schneewind *et al.,* 1992). The portion of the gene encoding the Fc-binding region is composed of five 160 bp repeats. The X-region of the gene contains a varying number of 24 bp repeats (Guss *et al.,* 1984; Uhlén *et al.,* 1984). The X-region is highly polymorphic, and has been used to discriminate between different human *S. aureus* strains (Frénay *et al.,* 1994; Shopsin *et al.,* 1999; Tang *et al.,* 2000). For example, Shopsin *et al.* (1999) studied 320 human *S. aureus* isolates. *spa* typing identified 33 different strain types and 24 distinct repeat types. They concluded that *spa* typing gave no further discrimination over PFGE, but that *spa* sequencing is a rapid typing tool for *S. aureus* that has advantages in terms of speed, ease of use, ease of interpretation, and standardisation among laboratories.

In the present study, PCR amplification of the X-region of the protein A gene gave a single band product, but the size of the band (0.3-0.6 kb) was able to identify and differentiate the avian, human and animal *S. aureus* strains into five groups (**'A'-'E'**) (**Table 3.17**). Restriction of the PCR product by *RsaI* (**Tables 3.17** and **3.18**) gave no further discrimination. According to these results, 72 (83%) of avian *S. aureus* isolates had similar patterns. All of the N. Ireland avian *S. aureus* isolates related to PFGE type 24, and those closely-related, possibly-related and some of the unrelated strains were undistinguishable. Another group of unrelated strains however gave different profiles. Comparison of the protein A typing with other typing methods shows that the protein A typing does not have the discrimination power of PCR-ribotyping method 1, REP-PCR or the coagulase gene RFLP analysis (**Tables 3.12** and **3.16**).

Isigidi *et al.* (1990) suggested that the absence of protein A differentiates *S. aureus* of the poultry biotype from the slaughterhouse biotype. On the other hand, Kusunoki *et al.* (1990) reported that strains from diseased chickens produce extracellular low-molecular-mass protein A. In a study by Rodgers *et al.* (1999), protein A was not present in some strains isolated from broiler parent farm personnel but was in all strains isolated from hatchery personnel.

The present study examined for the presence of the protein A gene in forty poultry and non-host specific biotype avian strains from N. Ireland (both typical and atypical) even though it was suggested that these did not express protein A, according to scheme of Isigidi *et al.* (1990) (**Tables 2.7** and **3.1**). According to our PCR method, all of them had at least the X-region of the protein A gene (**Table 3.17** and **Figure 3.17**). It may be that avian *S. aureus* isolates have the *spa* gene but they are not able to express it or only express it under certain conditions. The present study suggests that expression of the protein A gene in avian strains should be investigated further.

4.4.5 PCR amplification and RFLP analysis of virulence-regulatory genes

Two pleiotropically acting regulatory loci, *agr* and *sar*, have been identified that affect the temporal regulation of virulence gene expression in *S. aureus*. The product of the *sar* gene, SarA, is a DNA-binding regulatory protein that activates expression of the two operons in the *agr* locus. One operon expressed from the P2 promotor encodes a quorum-sensing system that effects the switch for surface protein expression when the population density is high, which occurs in later stages of infection or stationary phase laboratory cultures. The other operon in the *agr* locus is expressed from the P3 promotor. The most important product of its expression is a regulatory RNA molecule (RNAIII) that directly upregulates the expression of many exoproteins such as α, β and δ-haemolysins, toxic shock syndrome toxin-1, enterotoxins B and C, fatty-acid-modifying enzyme, lipase, serine protease, staphylokinase and serotype 5 capsular polysaccharide. Also

RNAIII downregulates the expression of some exoproteins such as protein A, fibronectin-binding proteins A and coagulase (Lee, 1998; Rechtin *et al.,* 1999).

Although the *agr* locus is conserved throughout staphylococci, the autoinduction circuit, consisting of signal receptor AgrC, the autoinducing peptide (AIP) processed from AgrD, and the putative AIP processing-secretion protein AgrB, shows striking variation, which serves to define distinct specificity groups (Ji *et al.,* 1997). Within any one group, these sequences are highly conserved and mutual cross-activation is observed. Between groups, however, there is mutual cross-inhibition of the *agr* signalling pathway (Ji *et al.,* 1997), giving rise to a novel type of bacterial interference that may have a role in colonisation and/or infection. In an earlier study (Ji *et al.,* 1997), three distinct *agr* specificity groups (I, II and III) of *S. aureus* strains were defined on the basis of mutual cross-inhibition of *agr* function, and Jarraud *et al.* (2000) demonstrated a fourth *agr* specificity group among human *S. aureus* strains and showed that most exfoliatin-producing strains belong to this group. A striking common feature of group IV strains is activation of the *agr* response early in exponential phase, at least 2 h earlier than in strains of the other groups. This finding by Jarraud *et al.* (2000) raises the question of the biological significance of the *agr* autoinduction threshold. Also Ji *et al.* (1997) have shown that most menstrual toxic shock human strains belong to *agr* specificity group III but that no strong clinical identity has been associated with strains of the other two groups.

Results from the present study are the first demonstration of *agr* and *sar* in avian *S. aureus* strains. It may be concluded that many of virulence factors characterised in this study in avian *S. aureus* are likely to be regulated by the *agr* and *sar* loci (*agrP2*, *agrBDC* and *sarA*) (**Figures 3.17**, **3.18**, **3.19** and **3.25**, **Table 3.21**). The results of the *agrBDC* region gene RFLP analysis and sequencing of these regions in selected strains suggested that 80% of avian *S. aureus* isolates belonged to *agr* specificity group II (auto inducing peptide [AIP]). Isolates in this group included the N. Ireland strains related to PFGE type

24, closely-related, possibly-related, and some unrelated (typical avian) strains, as well as Scottish, English and Sri Lankan isolates. On the other hand, 17% of the avian *S. aureus* isolates belong to *agr* specificity group I. Isolates in this group included some of the N. Ireland atypical strains, unrelated PFGE type 24, and two of the American isolates. 6% of the avian *S. aureus* isolates were found to belong to *agr* specificity group III. Of the four human *S. aureus* in which the *agrBDC* locus was restricted by *MseI*, two isolates were included into each of specificity groups I and III. In conclusion, typical avian *S. aureus* isolates were classified into specificity group II and atypical avian *S. aureus* isolates were classified into specificity groups I and III and this may affect their ability to produce many of the known virulence factors of *S. aureus*. However, there is no information concerning variations of *agr* and *sar* loci in avian *S. aureus* so further work is required to investigate this suggestion.

4.4.6 Development of a multiplex PCR for enterotoxins A-E, exfoliative toxins A –B and toxic shock syndrome toxin-1 (TSST-1)

In this study, multiplex PCR was developed and shown to be effective for the detection of genes encoding enterotoxins A-E (*sea* to *see*), exfoliative toxin A-B (*eta* and *etb*) and toxic shock syndrome toxin-1 (*tst*) genes. At the time this was done, there was no report of a multiplex PCR for all eight toxin genes. To give optimal resolution of each of the toxin genes, the concentration at which each primer was used was ten picomoles for *sea, seb, sec, see* and *tst*, 20 picomoles for *sed*, 30 picomoles for *etb* and 50 picomoles for *eta*.

PCR has been used for over a decade for the detection of these eight individual staphylococcal toxin genes (Neil *et al.,* 1990; Johnson *et al.,* 1991; Sakurai *et al.,* 1995; Jarraud *et al.,* 1999; Mehrota *et al.,* 2000; Sharma *et al.,* 2000). However, a few groups have recently developed multiplex PCRs for the simultaneous detection of some of the toxin genes (Becker *et al.,* 1998; Schmitz *et al.,* 1998; Jarraud *et al.,* 1999; Mehrotra *et al.,* 2000; Sharma *et al.,* 2000). For example, Schmitz *et al.* (1998) used a multiplex PCR for the detection of *seb, sec* and *tst*.

The PCR conditions were different from those in the present study in that each step had a shorter time with 20 seconds for amplification, 20 seconds for annealing and 50 seconds for extension in contrast to this study which allowed 30 seconds for denaturation, 30 seconds for annealing and one minute for extension. The primers were also different and gave different sized products for both *seb* (231 base pairs) and *tst* (250 base pairs), compared to 163 base pairs for *seb* and 445 base pairs for *tst* in this study. Becker *et al.* (1998) developed separate multiplex PCRs which detected all eight toxins *sea-see*, *tst, eta* and *eab*, with one multiplex detecting five genes (*sea, seb, sec, sed, see*) and another multiplex screening for three genes (*tst, eta* and *etb*). Some of the primers used by Becker *et al.* (1998) were different from those used in this study. For the PCR conditions, the same number of cycles was used as in this study, as well as the temperatures of each stage. The timing of each stage, however, was different from the present study i.e. with one minute for denaturation, one minute for annealing and two minutes for polymerisation. The advantage of having shorter timing of each segment means that the PCR cycle can be completed more quickly, which is an important factor in clinical diagnosis. Sharma *et al.* (2000) reported a multiplex PCR assay which specifically detects genes for five staphylococcal enterotoxins (A to E) in strains of toxigenic *S. aureus* from various environmental sources. The PCR conditions were different from those in the present study, with 30s for denaturation at 94 °C, 30s for annealing at 50 °C and 30s for extension at 72 °C. Jarraud *et al.* (1999) and Mehrotra *et al.* (2000) each developed two separate multiplex PCRs for amplification of these eight toxins, but again the conditions were different and the programmes were longer than the this study. In the present investigation all avian and some of the human and animal strains were screened for the eight toxin genes. Only 5% (4/86) of the avian *S. aureus* isolates but 53% (9/17) of the human *S. aureus* were positive for one or more of these genes. These findings by molecular methods were confirmed by biological methods for toxin detection.

In contrast, a number of previous studies have reported that a fairly high proportion of *S. aureus* strains isolated from birds produce enterotoxins or contain enterotoxin genes. For example, Harvey *et al.* (1982) found that 25% (35/139) of *S. aureus* isolates from raw poultry carcasses were enterotoxigenic. The majority of these strains (32/35) produced enterotoxin D alone. Only two isolates produced enterotoxin A and one isolate had enterotoxins C+D. Of those strains found to be enterotoxigenic, 91.4% were of the intermediate biotype with the cultural characteristics of human and animal types (Harvey *et al.,* 1982).

From the results of Šimkovicová and Gilbert (1971), Wieneke (1974), Payne and Wood (1974), and others, many avian strains of *S. aureus* of different phage groups or non-typable strains are reported to be capable of producing enterotoxins, but the majority of strains isolated from foods implicated in food poisoning incidents are human phage typable. This may be a result of either the ability of strains of these strains to produce higher levels of enterotoxins in foods or to have a higher growth rate in foods than other strains, or possibly the ´selective` recontamination of processed foods with strains from human food handlers. Lukášová *et al.* (1986) found that 45 strains of *S. aureus* isolated from slaughtered poultry were classified as biotype A (of human origin), according to the biochemical identification scheme of Hájek and Maršálek (1971), and 20 strains were biotype B (of poultry origin). Of the 20 biotype B strains, only one strain produced enterotoxin B. Of 22 biotype A strains, 12 (54.5%) strains produced different enterotoxins.

In another study, Gibbs *et al.* (1978a) showed the results of screening 55 *S. aureus* strains isolated from broiler and end-of-lay hen processing plants for the production of enterotoxins A, B and C. Overall, 62% of strains produced enterotoxins, enterotoxin A being by far the most common. Genigeorgis and Sadler (1966) found that 50% of the strains they isolated from the livers of commercially slaughtered poultry were enterotoxigenic. Wieneke (1974) reported that from frozen raw chickens 26% were enterotoxigenic, but none

produced enterotoxin A. From cooked chicken, 32% of strains produced enterotoxins and 7.6% produced enterotoxin A, and these strains were thought to be of human origin (Wieneke, 1974).

Sharma *et al.* (2000) used two single-reaction multiplex PCR assays to determine the toxin types of randomly selected *S. aureus* strains of ovine (67 strains), bovine (11 strains), poultry (40 strains), and human (39 strains) origins. The poultry strains were from various sources within a poultry processing plant (both environmental and carcasses). In parallel, the strains were also tested by staphylococcal enterotoxin reversed passive latex agglutination (SET-RPLA) detection kit. The results showed that 31% of human strains produced different enterotoxins and 9% of bovine strains produced enterotoxin C. 75% of ovine strains produced different enterotoxins and 50% of poultry strains were toxin positive (enterotoxin D). The toxin serotype results agreed with the PCR method, except that two ovine isolates were negative with PCR method, but they were positive with SET-RPLA.

The conclusion from this published work is that some strains isolated from poultry do appear to produce enterotoxins but most of these strains are from processed birds and may therefore have been contaminants from the processing plant equipment or from human handlers. This suggestion is supported by the findings of biotyping studies where this has been done. Most of the enterotoxin-producing isolates were non-poultry biotypes.

The four avian *S. aureus* strains in this study that were found to produce toxins were not typical avian strains in that they gave a positive reaction with PCR-ribotyping method 2. Isolate 32528 was shown to contain the *tst*, enterotoxin A and exofoliative toxin B genes. This strain was obtained from a Cockatoo. There has been no previous reports that avian *S. aureus* strains possess *tst* and exofoliative B genes and it may be this isolate was of human origin. Three of the four strains produced enterotoxin A. Strains 496 and 525 were identified as the

non-host specific biotype and strains 31178 and 32528 were not biotyped. All of these isolates according to PCR-ribotyping method 2 and RFLP analysis of the *agrBDC* region had band patterns similar to human strains (**Table 3.19**). For these reasons, it is possible that these strains may be of human origin. The present study concluded that 33.3% (4/12) of atypical avian *S. aureus* strains, but none of the typical avian strains, possessed one of more of these eight toxin genes.

Results on the human *S. aureus* strains in this study showed that 53% of strains produced one or more of the eight toxins. Becker *et al.* (1998) found that 42% of clinical isolates of MSSA were found to be toxin positive for one or more of these eight toxins by PCR. Similarly, Neil *et al.* (1990) found 59% of clinical isolates to be toxin positive for one or more of these eight toxins by PCR. Adesiyum *et al.* (1992) who used ELISA to detect *sea*, *seb*, and *sec* found 68% of isolates from human wounds and diarrhoea to be toxigenic. Taken together these results show that human strains commonly produce these toxins.

4.4.7 PCR amplification of haemolysin genes and haemolysin production

There is no previous report describing the detection of haemolysin genes in avian *S. aureus* or other avian staphylococci species by molecular methods, nor is there any report of a multiplex PCR for detection of α, γ and δ-haemolysin genes in avian, human or animal *S. aureus* strains.

The multiplex PCR results for haemolysin (α, γ and δ) genes in the present study showed that all the 86 avian *S. aureus* isolates had the α-haemolysin gene. The biological results for α-toxin production agreed largely with the molecular method as 84 (97%) were α-haemolytic. Only two isolates, 82 and 83 (atypical avian isolates), did not show haemolysis on blood agar but were positive with the molecular method. These results are in agreement with previous reports. For example, Gibbs *et al.* (1978a) showed that 100% of *S. aureus* strains isolated from five broiler processing plants and from one plant processing end-of-lay

hens produced α-haemolysin. Also, in the study by Gibbs *et al.* (1978b), 83% of *S. aureus* strains isolated from processed poultry and processing plants produced α-haemolysin. Thompson *et al.* (1980a) reported that 88% of *S. aureus* strains isolated from live-birds produced α-haemolysin. Harvey *et al.* (1982) showed that 100% of *S. aureus* strains isolated from raw poultry carcasses produced α-haemolysin.

Of thirteen human *S. aureus* isolates examined in this study by molecular methods for the haemolysin genes, all gave a positive PCR reaction for α-haemolysin. Of the three avian coagulase-negative strains tested, none of them showed haemolysins genes. Of the four animal *S. aureus* isolates examined, all isolates had the α-haemolysin gene. Christensson and Hedström (1986) reported that, in human isolates of *S. aureus*, 58% of strains produced α-haemolysin. Coia *et al.* (1992) determined haemolysin produced by methicillin-resistant (MRSA) and sensitive (MSSA) *S. aureus*. According to their results, the distribution of α-haemolysin production was 81% and 65% for MRSA and MSSA respectively. Aarestrup *et al.* (1999) showed that 63% of human isolates of *S. aureus* and 37% of bovine *S. aureus* isolates were α-haemolytic.

The multiplex PCR results showed that all the 86 avian *S. aureus* isolates also had the δ-haemolysin gene. Gibbs *et al.* (1978a) showed that 100% of *S. aureus* strains isolated from five broiler processing plants and from one plant processing end-of-lay hens produced δ-haemolysin. In another study by Gibbs *et al.* (1978b), 98% of *S. aureus* strains isolated from processed poultry and processing plants produced δ-haemolysin. Thompson *et al.* (1980a) reported that 45% of *S. aureus* strains isolated from live-birds from different countries produced δ-haemolysin. The haemolysin results by the molecular method for δ-haemolysin in the present study on avian *S. aureus* strains agreed with the biological method in the studies of Gibbs *et al.* (1978a, b).

Of thirteen human *S. aureus* isolates examined in this study, all had the δ-haemolysin gene. According to the results of Coia *et al.* (1992) 62% and 61% of MRSA and MSSA respectively produced δ-haemolysin. In another report, δ-haemolysin was produced by 97% of human *S. aureus* isolates and also by 50-70% of coagulase-negative staphylococci (Bohach *et al.*, 1997).

There is no published information about γ-haemolysin production in avian isolates of *S. aureus*. The multiplex PCR results in the present study showed that 74 (86%) the 86 avian *S. aureus* isolates had the γ-haemolysin gene. Of the twelve strains that did not appear to have the γ-haemolysin gene, eight of these according to PCR-ribotyping method 2 and other molecular methods were in the atypical avian group. These isolates were 82, 83, 86, 496, 525, 562, 217 and 235 (**Table 3.20**). Two of the four other strains did not have γ-haemolysin were in another group of atypical avian *S. aureus* strains. These isolates were NFR (from bumbelfoot in a Gyr falcon) and 31039 (from skin scab lesions in a commercial flock) (**Table 3.21**). Another two avian strains that did not have γ-haemolysin were PD458 from Sri Lanka (typical avian) and 98 from N. Ireland (typical avian) unrelated to type 24).

Of thirteen human *S. aureus* isolates examined in this study, 61.5% of the isolates showed a γ-haemolysin band. Christensson and Hedström (1986) reported that, in human isolates of *S. aureus*, 24% of strains produced γ-haemolysin. Coia *et al.* (1992) determined haemolysins produced by methicillin-resistant (MRSA) and sensitive (MSSA) *S. aureus*. According to their results, the distribution of γ-haemolysin production was 69% and 57% for MRSA and MSSA respectively. Bohach *et al.* (1997) reported that nearly all human *S. aureus* strains manufacture γ-haemolysin, which is inactivated by agar and therefore non-demonstrable on blood agar plates.

The PCR results for detection of the β-toxin gene in this study showed that only four of the 86 avian *S. aureus* isolates (5%) gave a positive reaction. According to PCR-ribotyping method 2 and other molecular methods, these strains were in the atypical avian groups (**Tables 3.20**, **3.21** and **3.23**). These strains were 31178, 32528, 496 and 525. The biological assay for β-toxin in the present study agreed with the molecular method for all 86 strains except that two avian *S. aureus* isolates, 31039 and 562 gave a positive result with the biological method but did not give a positive result with the molecular method. These two strains according to other molecular methods were in an atypical avian group (**Tables 3.20**, **3.21** and **3.23**). Sequence variation in the primer regions in the β-haemolysin encoding gene of isolates 31039 and 562 is suggested, and should be investigated further.

Gibbs *et al.* (1978a) showed that 18% of *S. aureus* strains isolated from five broiler processing plants and one plant processing end-of-lay hens produced β-haemolysin. In another study, Gibbs *et al.* (1978b) showed that 24% of *S. aureus* strains isolated from processed poultry and processing plants produced β-haemolysin. On the other hand, Thompson *et al.* (1980a) reported that <1% (1/108) of *S. aureus* strain isolated from live birds from different countries produced β-haemolysin. Harvey *et al.* (1982) showed that none of *S. aureus* strains isolated from raw poultry carcasses produced β-haemolysin. Rodgers *et al.* (1999) reported that none of the *S. aureus* isolated from skeletal disease in broilers produced β-haemolysin. Also, early work on staphylococci isolated from poultry has shown wide variations, from 0-36%, in the proportion of strains producing β-lysin (Genigeorgis and Sadler, 1966; Harry, 1967a, b; Devriese *et al.*, 1972; Sato *et al.*, 1972).

Of the two avian coagulase-negative strains examined for β-haemolysin, neither showed any band. Of the twelve human *S. aureus* isolated tested for β-haemolysin, seven (58%) showed a β-haemolysin band. Coia *et al.* (1992) had shown β-haemolysin was produced by 23% and 35% of MRSA and MSSA,

respectively. According to other reports, 10-15% of *S. aureus* human isolates and 75-100% of bovine isolates were β-haemolytic (Elek and Levy, 1950; Poutrel and Ducelliz, 1979). Similarity Aarestrup *et al.* (1999) found the β-haemolysin gene in 100% of bovine *S. aureus* and in 56% of the human isolates examined. Larsen *et al.* (2002) showed that 97% of *S. aureus* strains isolated from bovine mastitis in Europe and USA were PCR-positive for and/or produced β-haemolysin on 5% calf blood agar. Except for three isolates, the Norwegian isolates were PCR-negative, but positive on 5% calf blood agar.

Overall, it seems that β-haemolysin production is common in human and especially bovine strains of *S. aureus* but uncommon in typical avian strains. It may be that the four atypical avian strains identified in this study as possessing the β-haemolysin gene and two additional strains producing β-haemolysin on blood agar were originally from human or animal sources.

4.5 SEQUENCE ANALYSIS

DNA sequence analysis is an objective genotyping method and the resulting sequence is highly portable and easily stored and analysed in relevant databases. Recent advances in DNA sequencing technology, including rapid, affordable, high-throughput systems, have made it possible for sequencing to be considered as a viable typing method. Sequencing the same DNA targets from different isolates and then cataloguing the allelic patterns is called comparative sequencing. Two different strategies have been used to provide genotyping data: multilocus sequence typing (MLST), which compares sequence variation in numerous housekeeping gene targets, and single-locus sequence typing, which compares sequence variation of a single target.

In the present study sequence analysis of a number of selected genes was determined, for typical and atypical avian and some control *S. aureus* strains.

4.5.1 Sequence analysis of 16S rRNA gene

Comparison of 16S rRNA sequences has proved extremely useful for determining phylogenetic relationships among eukaryotic and prokaryotic organisms (Woese *et al.,* 1985; Woese, 1987). Dewhirst *et al.* (1992), using 16S rRNA sequence comparisons, demonstrated that *Pasteurella haemolytica* and *Pasteurella trehalosi* belong to different clusters in the family Pasteurellaceae and may, in fact, belong to different genera. However, it should be noted that both *P. haemolytica* and *P. trehalosi* were excluded from the genus *Pasteurella* sensu stricto by Mutters *et al.* (1985), and *P. haemolytica* has since been reclassified as *Mannheimia haemolytica*. In the present study 16S rRNA sequences were examined for any differentiation between the *S. aureus* isolates. Sequence analysis of 16S rRNA gene of *S. aureus* isolates including seven avian and one human strains showed that all were 100% identical and also identical to that of another human *S. aureus* strain ATCC 12600 published in GenBank (accession number L37597). Thus there was no evidence from 16S sequences that avian strains form a group distinct from human strains.

4.5.2 Sequence analysis of coagulase gene region

The present study reports the sequence of the coagulase gene from avian *S. aureus* strains. As described in section 3.9.2, sequence analysis of the repeat region of the coagulase gene showed that typical avian *S. aureus* isolates 24, 85, 99, 2B, 31174, PD27, 34 and 38 were 100% identical, and they were 97% (nucleotides) and 100% (amino acids) identical to that of a human *S. aureus* isolate 213 in GenBank (accession number X16457). The typical avian *S. aureus* isolates 24, 85, 99, 2B, 31174, PD27, 34 and 38 were 95% (nucleotides) and 94% (nucleotides) identical to atypical avian *S. aureus* strains 82 and 496, respectively. These results indicate a relationship between RFLP analysis of the coagulase region and sequence analysis for typical avian *S. aureus* (**Table 3.15** and **Figures 3.15a** and **b** and **Appendix III-b**). Sequencing results of atypical avian isolates 82 and 496 also show that they were 94% and 95% identical with a human *S. aureus* strain 213 in GenBank (accession number X16457). Isolates

82, 83, 86, 562, 217 and 235 were 98.5% (nucleotides) identical to strains 496 and 525. However, the sequence of human isolate RN4220 was only 94% (nucleotides) identical to that of human isolate BB in the database.

From sequence analysis of the coagulase repeat region it can concluded that typical avian *S. aureus* isolates are different from atypical avian or human *S. aureus* isolates, but more isolates would need to be compared before a clear relationship could be discussed.

4.5.3 Sequence analysis of *agrBDC* gene region

Sequence analysis of the *agrD* gene within the *agrBDC* gene region was used to show variation in autoinducing peptide production in *S. aureus* strains. Typical avian strains 24, 85, 99, 2B, 31174, PD27, 104, 595, 35, 98, 34 and 38 and atypical avian strains 82, 83, 86, 562, 217, 235, 496, 525, 31178 and 32528 had different sequences and the sequence of strains 82, 83, 86, 562, 217 and 235 was different from that of strains 496, 525, 31178 and 32528. The sequence also revealed that the typical avian strains 24, 85, 99, 2B, 31174, PD27, 104, 595, 35, 98, 34 and 38 were in group II of the autoinducing peptides encoded by *agrD* (Jarraud *et al.,* 2000). Strains 82, 83, 86, 562, 217, 235 and RN4220 (human strain) were in group I and strains 496, 525, 31178 and 32528 were in group III according to this classification scheme (Jarraud *et al.,* 2000).

The biological role and significance of these AgrD groupings is not clear, but group-specific differences in the expression of subsets of virulence factors or other extracellular proteins could be possibly related to differences in disease patterns. Atypical avian strains 31178, 32528, 496 and 525 were in group III and produced one or more of enterotoxins A and B, exfoliative toxin B and toxic shock toxin-1. Ji *et al.* (1997) have shown that most human *S. aureus* strains that produced toxic shock toxin-1 were in *agr* specifity group III, but Jarraud *et al.* (2000) showed that most exfoliative toxin-producing human *S. aureus* strains

belong to group IV. However, the present study concluded that the sequence of *agrD* can be used to differentiate typical avian from some atypical avian isolates.

4.6 EXTRACELLULAR PROTEINS

4.6.1 Exoprotease activity in avian strains of *S. aureus*

At least four major extracellular proteases have been reported to be produced by strains of *S. aureus* (Arvidson, 2000): staphylococcal serine protease (V8 protease) (SspA), a metalloprotease named aureolysin (Aur), a cysteine protease (*Scp*) named staphopain (Hofmann *et al.,* 1993), and a second cysteine protease (SspB) encoded within the same operon as SspA (McGavin *et al.,* 1997; Chan and Foster, 1998). All four proteases appear to be synthesized as preproenzymes, which are proteolytically cleaved to generate the mature enzymes (Drapeau, 1978; Rice *et al.,* 2001). The synthesis of extracellular proteases is positively regulated by *agr* and negatively regulated by SarA (Janzon *et al.,* 1986; Chan and Foster, 1998). The gene for a second serine protease was identified in whole genome sequencing (Kuroda *et al.,* 2001) and this protease may have a function similar to V8 protease (SspA). Genes encoding metalloproteases have been cloned from *S. hyicus* (Ayora and Götz, 1994). Takeuchi *et al.* (2000) reported a common metalloprotease in swine, avian and bovine isolates of *S. hyicus* and *S. epidermidis* (Teufel and Götz, 1993), with the metalloprotease from *S. epidermidis* exhibiting elastase activity. Recently in GenBank (January, 2001), Dubin reported the sequence of an extracellular cysteine proteinase in human *S. epidermidis* isolate 6746 (GenBank accession number AJ298299).

At the start of the present work there was little published information on protease activity in avian strains of *S. aureus*. Kuramasu *et al.* (1967a) isolated strains of *S. aureus* which had strong proteolytic activity from chickens suffering from edematous and necrotic dermatitis. In addition, they conjectured that there might be a relationship between the protease and the dermatitis in the chickens,

because dermatolysis was observed in young chickens and mice when they were inoculated subcutaneously with more than 10^7 cells of a protease-positive strain. More recently, Takeuchi *et al.* (1999) characterised two proteases produced by an avian strain of *S. aureus*, from a chicken suffering from dermatitis. The two proteases, of molecular weight 23.1 and 22.7 kDa, were purified from the culture supernate of this strain. Their isolelectic points were 5.85 and 5.55 respectively, they had antigenic similarity and their N-terminal amino acid sequences were identical: RAQYVNQLKNFKIRETQ. Their activity was strongly increased by reducing agents such as L-cysteine, they were inhibited by thiol (cysteine) protease inhibitors but not by metalloprotease or serine protease inhibitors and were thus presumed to be thiol (cysteine) proteases (Takeuchi *et al.,* 1999).

One of the most striking findings in the present study was the presence of a single dominant protein band in the culture supernates of most avian *S. aureus* strains. The results from SDS-PAGE gels in this study on 67 avian *S. aureus* isolates showed that 60 (89.5%) of these isolates had a single dominant protein band of 22 kDa (**Figure 3.5**). N-terminal sequence analysis of the 22 kDa protein provided the first 11 amino acids, the sequence of which, VRAQYVNQLKN, was almost identical to that reported for the thiol (cysteine) proteases studied by Takeuchi *et al.* (1999). This finding suggested how the 22 kDa protein could be further characterised. Screening on skim milk agar plates and the effect of different inhibitors and an activator on protease activity supported the conclusion that the 22 kDa band represented a cysteine protease. Amplification and sequencing of a 522 bp region from avian *S. aureus* isolates 24 and 31179 and from the human *S. epidermidis* isolate 134 showed that the sequences for strains 24 and 31179 were 100% identical but had only 80% identity to that in the *S. epidermidis* strain and 42% identity to the cysteine protease (staphopain) gene in human *S. aureus* isolates. The corresponding sequence of the *ecp* gene of human *S. epidermidis* isolate 134 used in the present study showed 100% identity with the published sequence (**Appendix III-a**). When amino acid sequences were compared the avian cysteine protease had

81% identity to the extracellular cysteine proteinase (*ecp*) in *S. epidermidis*. 77% identity to the *scp* cysteine protese (staphopain) gene in human *S. aureus* strains and 50% identity to the second cysteine protease (*sspB*) gene in human *S. aureus*.

Of the 67 avian strains examined, 60 strains had the 22 kDa band in the culture supernate and showed protease activity on skim milk agar. It seems likely therefore that all of these strains produced a cysteine protease. All of these strains were from diseased birds. 38 (65%) isolates were from broilers with different diseases such as synovitis; femoral head necrosis; tibial dischondroplasia; rotated tibia; bumblefoot and proximal femoral degeneration and the site of isolation were joint, foot, and a case from skin. Eight (13.3%) of the 60 isolates were from chicks, 1-2 days old, with different diseases such as yolk sac infection and mushy chick disease and the sites of isolation were yolk sac and heart. For the other isolates, these was no record about disease history or site of isolation. The other seven isolates did not have a 22 kDa protease band in their culture supernates and were negative for protease activity on skim milk agar (except isolate 31177 which was positive on skim milk agar). Four of these isolates, 31178, 32528, 82 and 496, showed bands with PCR-ribotyping method 2, and thus are atypical avian isolates. Two other isolates that did not give positive-protease bands were 31039 (from skin scab lesions) and K (from bumblefoot). Again these strains were atypical avian *S. aureus* according to different molecular methods. Strain 31177 was typically avian by molecular methods. It is tempting to speculate that the extracellular cysteine protease produced by disease-causing avian isolates might play a role in joint, foot and yolk sac diseases in birds. Further work is required to confirm this suggestion. One way to do this would be to create "knock-out" mutants in the protease gene and to compare the virulence of the wild-type and mutant strains in different models of infection. For example, intravenous injection of staphylococci can be used to produce chronic synovitis in chicks (Forget *et al.*, 1974).

This study also concluded that human *S. epidermidis* isolates have the cysteine protease gene but they may not be able to express it under the in vitro growth conditions used here (**Figures 3.6 and 3.10**). Protease activity on gelatin gels showed slightly different patterns between strains of PFGE type 24 (**Figure 3.11**) and it may be possible to improve this method for discrimination between avian *S. aureus* strains related to PFGE type 24 according the designation of McCullagh *et al.* (1998).

4.7 GROWTH OF *S. aureus* STRAINS AT HIGH TEMPERATURES

These experiments need to be repeated with a wider selection of strains and with a more accurate temperature control over say 44-47 °C. In this way, it may be possible to distinguish strains that are well adapted to growth in birds with their higher body temperatures and those strains that have been acquired more recently from other sources.

4.8 SUMMARY OF TYPING OF AVIAN STRAINS

One focus of interest in the present study was to examine a collection of avian *S. aureus* strains that had been biotyped and typed by PFGE according to the scheme of McCullagh *et al.* (1998), and had been shown to be either indistinguishable or related or unrelated by these methods. This collection of strains that were "type 24", "closely-related" (CR), "possibly-related" (PR), or "unrelated" (UR) to type strain 24 on the basis of their PFGE band patterns were kindly provided by Dr. McCullagh (Veterinary Science Division of Agriculture for Northern Ireland, Belfast). These strains were subjected to the various molecular fingerprinting methods developed in this study and a summary of the results obtained with the genotyping methods is shown in **Table 3.20**.

All of the type 24 strains (i.e. indistinguishable from type strain 24 by PFGE) gave identical profiles by the panel of tests listed in the table and so these tests

were no more discriminating than PFGE. Strain 77 was different from the other type 24 strains by plasmid profiles (**Table 3.4**), antibiotic resistance pattern (**Table 3.9**) and 22 kD protease band (**Table 3.10**). Strains 572 and 582 showed differences by antibiotic resistance pattern (**Table 3.9**) and on gelatin gels (**Figure 3.11**). Strain 100 was differentiated by its antibiotic resistance pattern (**Table 3.9**). Strains 85 and 123 were differentiated on the gelatin gels (**Figure 3.11**). The majority of the type 24 strains that had been biotyped belonged to the poultry group. Four type 24 strains (11, 31, 33, 47) belonged to the NHS biotype i.e. they differed from the poultry biotype in **Table 3.1**. None of the molecular methods listed in **Table 3.20** or other tests were able to discriminate between these two biotypes.

Four of the strains examined had been designated as CR by McCullagh *et al.* (personal communication) because their PFGE profiles showed (1-3) band differences from the type 24 profile. One such CR strain (34) had the NHS biotype and two (38 and 564) had the poultry biotype but were indistinguishable by the methods listed in the Table. Strain 564 was different from the other CR strains by plasmid profiles (**Table 3.4**) and antibiotic resistance pattern (**Table 3.9**). One CR strain (595) and the only PR strain available (i.e. differing from type 24 in (4-6) bands by PFGE), however, were distinguishable from all of the above strains in that they had distinct profiles by PCR ribotyping method 1 and after restriction of the coagulase gene amplimer and also by plasmid profiles (**Table 3.4**) and antibiotic resistance pattern (**Table 3.9**). In these properties, they resembled a group of UR strains (see below) but could be distinguished from them by REP-PCR. Thus the genotyping methods used in present study were able to distinguish one CR strain from three others. The CR strains differ from type 24 in their PFGE profiles but it is not known if they differ from each other by this method. Unfortunately, no records of their individual profiles were available from Dr McCullagh. It would be of interest, therefore, to repeat the PFGE typing with these and other selected strains to compare this typing method with the methods developed in the present study.

One group of strains listed in **Table 3.20** (strains 35, 36, 98, 580, 597, 598) and designated as UR (unrelated to type 24 and differing by more than 6 bands in their PFGE profiles) formed a distinct group that were identical by the genotyping methods used here but different from the above type 24 strains and 3 of the CR strains by ribotyping method 1, REP-PCR and their restricted coagulase gene PCR amplimer. According to McCullagh (personal communication) these strains are also related to each other on the basis of PFGE. Isolates 35, 580, 597, 598 were indistinguishable by PFGE, isolate 36 was closely-related to this group and isolate 98 possibly-related according to the PFGE patterns and the criteria used by McCullagh *et al.* (1998). Thus there was good agreement between the PFGE method and the methods used in the present study although the former was more discriminating. Strains 98, 597 and 598 could be differentiated among the UR strains by plasmid profiles (**Table 3.4**).

An interesting group of strains was another group (82, 83, 86, 562, 496, 525) of UR strains that, unlike most of the other avian strains, could be typed by ribotyping method 2. This ribotyping method had been developed for the identification of bovine isolates of *S. aureus* (Forsman *et al.,* 1997) but also gave amplimers with all 14 of the strains from human and diverse animal sources obtained in the present study. Three strains had the poultry biotype, two had the NHS biotype and one had the slaughterhouse biotype (see **Table 3.1**). This atypical group of avian isolates also differed from the other avian Northern Ireland strains in their profiles obtained by ribotyping method 1, REP-PCR, coagulase and protein A gene amplimers, after restriction of the *agrBDC* region and by their lack of the γ-haemolysin gene. Three strains (82, 83, 86) were indistinguishable by these means. Isolate 562 differed from these three strains only in the band patterns obtained for the protein A gene region, which was identical to those for the more typical avian strains and possibly by its production of β-haemolysin (biological method). The two NHS biotype strains 496 and 525 were different but indistinguishable from each other. According to McCullagh (personal communication) strains 82 and 83 were isolates from the

same broiler farm and were identical to each other by PFGE. They were unrelated in their PFGE type to isolate 86 but there was no difference between these strains by the fingerprinting methods used in the present study. Strains 496 and 525 had identical PFGE types and their genetic relatedness was confirmed by the methods used here, including their toxin and haemolysin genes. Isolate 562 was from a pheasant and had a unique PFGE type and was also distinct by the fingerprinting methods used here. Again, there was good agreement between the PFGE method and the methods used in the present study although the former was more discriminating. It would be of interest to repeat the PFGE typing with these strains to compare this typing method with the methods developed in the present study. Strains 82, 83, 86 and 562 were differentiated among this group of the UR strains by plasmid profiles (**Table 3.4**); also strains 86 and 562 had antibiotic resistance patterns different from other strains of this group (**Table 3.9**).

The majority (24/35) of the other avian isolates used in the present study, from different geographical locations, were indistinguishable, on the basis of the genotyping methods used in the present study, from the type strain 24 from Northern Ireland. Strains 3A, 6, 7A, 7B and PD458 were differentiated in this group of the strains by plasmid profiles (**Table 3.4**). Also, except for strains PD27, PD380, PD458 and PD486 that had similar antibiotic resistance patterns to the type 24 strains, other strains had different antibiotic resistance patterns from type 24 (**Table 3.9**). It appears, therefore, from the collection of strains used in the present study, that the majority of avian *S. aureus* isolates from diseased birds form a closely-related group, irrespective of geographic origin. This suggestion could be investigated by thorough taxonomic studies, for examples using MLEE and or MLST, as has been done for human isolates of *S. aureus* (Enright *et al.,* 2000).

Only 11 of the 35 avian strains that were not from Northern Ireland gave atypical profiles by the genotyping methods used here. The results for these

strains are shown in **Table 3.21** and they can be seen to fall into two broad groups depending on whether they were typable by ribotyping method 2. The strains that did not type by ribotyping method 2 were mostly very similar to the typical avian strains in that three of them differed in only one property i.e. strain 31036 differed only in the protein A gene region, 31039 differed by ribotyping method 1 and lack of γ-haemolysin gene (and possibly produce of β-haemolysin) and E differed only in the coagulase gene region. Strain K differed by ribotyping method 1 and in the protein A gene region whereas strain NFR differed in several properties.

The six strains in this group that typed by ribotyping method 2, like those in **Table 3.20**, were a heterogeneous group and also differed from typical strains by the other genotyping methods including in some cases their toxin and haemolysin gene profiles. Any future work on the above atypical isolates should also include PFGE typing according to the scheme of McCullagh *et al.* (1998) and biotyping according to the scheme of Devriese (1984) and Isigidi *et al.* (1990) in order to compare these methods with those developed in the present study. It could be predicted that they would be unrelated to type 24 and probably to each other by PFGE typing. Taxonomic studies by MLEE and MLST might reveal that the strains that type by ribotyping method 2 are only distantly related to the typical avian strains and might possibly be more closely related to human or animal strains. This last possibility is reinforced by a number of the fingerprinting methods used in the present study where these atypical strains have profiles which contain bands in common with some of the randomly collected human and animal strains used as controls in **Tables 3.13-3.19**. Also, the only avian isolates found to produce staphylococcal enterotoxins (e.g. strains 496, 525, 31178), TSST-1 and exfoliative toxin (strain 32528), were found in this group. These particular strains may have been recently acquired from human or animal sources (e.g. strain 32528, isolated from a cockatoo and therefore likely to be in contact with humans). Other strains in this group may have evolved from human or animal sources but adapted to the avian environment.

Some of them, at least, were known to have the poultry biotype. Of these strains, only 31036 and E had the 22 kDa protein band.

4.9 CONCLUSIONS

Results of this investigation have shown that the majority of avian isolates of *S. aureus* appear to be clearly distinct from *S. aureus* isolates from other host species and from avian coagulase-negative strains of staphylococci. For example, a high proportion (95%) of avian *S. aureus* isolates have one or more plasmids whereas only a low proportion of avian coagulase-negative and human *S. aureus* isolates possess one or more plasmids. In addition, the majority of avian isolates did not produce enterotoxins or β-haemolysin, yet they produced a large amount of the putative extracellular cysteine protease. By contrast, human isolates generally produced one or more enterotoxins and β-haemolysin, but not the exoprotease.

The clearest difference between avian *S. aureus* and *S. aureus* from other sources was provided by the molecular fingerprinting methods. The majority (80%) of the avian *S. aureus* strains, even from different geographical locations, had similar "typical avian" profiles whereas 20% had atypical avian profiles but, overall, these latter strains were still distinct from human and animal isolates. Thus, from the avian *S. aureus* strains collected in the present study, it appears that "typical" avian strains, like the type 24 strains described by McCullagh *et al.* (1998) or those strains indistinguishable from type 24 by the fingerprinting methods used here, are responsible for the majority of cases of disease in chickens. However, many more isolates from different sources would need to be collected and typed before this hypothesis could be proved. Comparative studies in a suitable avian or animal model of infection would indicate whether these different properties were reflected in differences in the manner by which avian and other *S. aureus* strains colonised and persisted in the host during infection.

4.10 FUTURE WORK

Many different lines of investigations are suggested by the findings in this study, for example:

1. The most important of these would be to investigate the role that the extracellular cysteine protease in avian *S. aureus* isolates might play in leg weakness (joint, foot) and yolk sac disease in birds. The aim would be to create "knock-out" mutants in the protease gene and to compare the virulence of the wild-type and mutant strains in different models of infection. Alternatively, the protease could be purified or produced by recombinant means and made into a vaccine for testing as a protective antigen.

2. Taxonomic studies by MLST and MLEE on a large number of avian, animal and human isolates would be interest. For example this might reveal that the avian *S. aureus* strains that type by ribotyping method 2, or are otherwise "atypical", are only distantly related to the typical avian *S. aureus* strains and might be more closely related to human or animal strains.

3. Other work could be to determine the correlation, if any, between antibiotic resistance and the presence of some of the common plasmids in avian *S. aureus* and avian coagulase-negative, and if these plasmids are transmissible between strains.

4. Expression of the protein A gene in avian *S. aureus* strains should be investigated. According to the biotyping scheme of Isigidi *et al.* (1990), the avian biotype does not express protein A. However, from the present work, the protein A gene is present in all avian strains.

5. APPENDICES

Appendix I

5.1 Media composition

5.1.1 Luria Bertani broth (LB)

1 Litre

Tryptone	10g
Yeast extract	5g
Sodium chloride	10g

Add 1.2% agar for solid media

5.2 Molecular biology solutions

5.2.1 Tris-borate-EDTA (TBE) buffer (5x stock solution)

Tris	54g
Boric acid	27.5g
EDTA (0.5M stock)	20ml

5.2.2 DNA loading dye (6x stock solution)

Tris	60mM
EDTA	6mM
Sucrose	40%
Bromophenol blue	0.25%

5.3 Protein analysis solutions

5.3.1 Protein sample buffer

Glycerol	5ml
20% SDS	2.5ml
2-mercaptoethanol	0.5ml
Tris (0.5ml, pH 6.8)	2.5ml
Bromophenol blue	0.25%

5.3.2 Stacking gel (6%)

Acrylamide/Bis solution	4ml
dH_2O	10.69ml
Tris-HCl (0.5M, pH 6.8)	5ml
20% SDS	100µl
TEMED	10µl
10% Ammonium persulphate (APS)	200µl

5.3.3 Separating gel (12.5%, adjust volume of Acrylamide/Bis solution and dH_2O \ to change percentage)

Acrylamide/Bis solution	4ml
dH_2O	12.6ml
Tris-HCl (0.5M, pH 6.8)	10ml
20% SDS	400µl
TEMED	30µl
10% Ammonium persulphate (APS)	300µl

5.3.4 PAGE running buffer (10x)

1 Litre

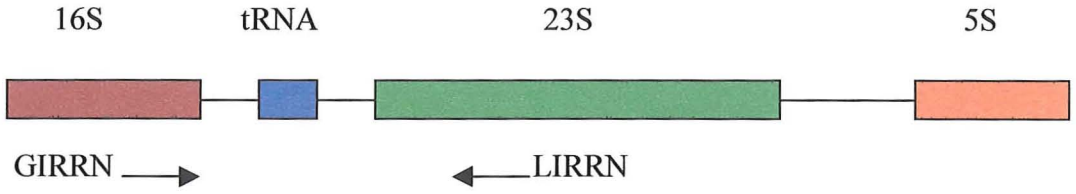
Tris	30g	
Glycine	144g	pH 8.3
SDS	10g	

5.3.5 Coomassie blue gel stain

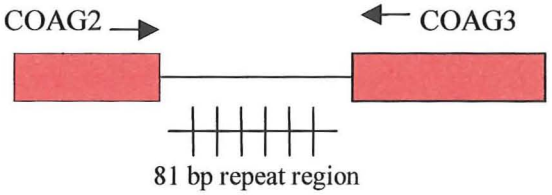
Coomassie blue	0.5g
Methanol	500ml
Acetic acid	100ml
dH_2O	400ml

For destain solution the Coomassie blue was omitted.

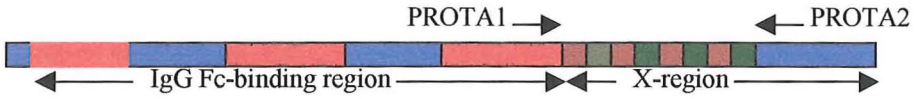
Appendix II Schematic representation of the amplification of different regions of the *S. aureus* genome. The approximate locations of the forward and reverse primers sites are shown.



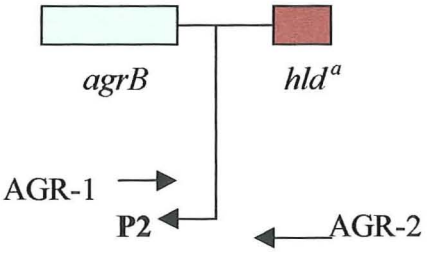
a) 16S-23S spacer region.



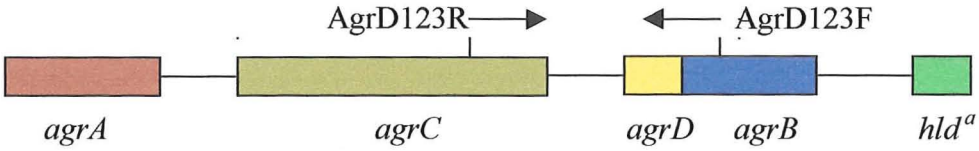
b) Region of 81 bp tandem repeats of the coagulase gene.



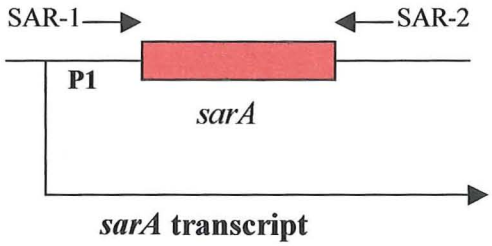
c) The X-region of the protein gene.



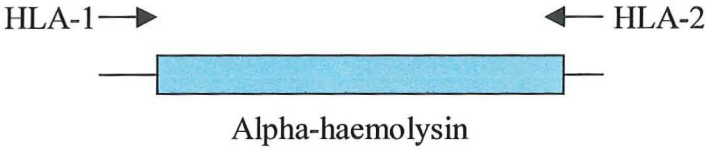
d) The *agrP2* region.
hld[a]: Delta-haemolysin



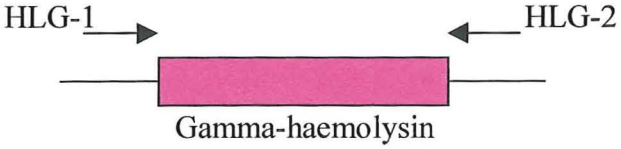
e) The *agrBDC* region.
hld^a: Delta-haemolysin



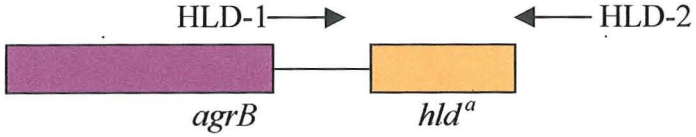
f) The *sarA* region.



g) The alpha-haemolysin gene.



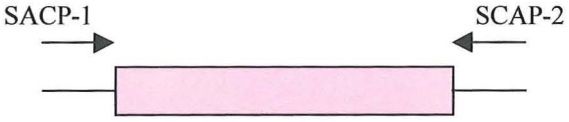
h) The gamma-haemolysin gene.



i) The delta-haemolysin gene
hld^a: Delta-haemolysin

Beta-haemolysin

j) The beta-haemolysin gene.



Cysteine protease

k) The cysteine protease gene.

Appendix III. Comparison of sequences of different genes.

a) Comparison of sequences of cysteine protease genes

```
[a]24              ATGAGAGCACAATATGTAAATCAATTAAAGAACTTTAAAATCAGAGAAACACAAGGTAAT 60
[a]31179           ATGAGAGCACAATATGTAAATCAATTAAAGAACTTTAAAATCAGAGAAACACAAGGTAAT 60
[b]134             ATGTACGCAGAATATGTAAATCAATTAAAGAATTTCAGAATACGAGAAACACAAGGGTAT 60
                   ***   *** ********************** ** * ***  *************  **

24                 AATGGTTGGTGTGCTGGATATACAATGTCAGCATTACTGAATGCAACTTATAATACAGAT 120
31179              AATGGTTGGTGTGCTGGATATACAATGTCAGCATTACTGAATGCAACTTATAATACAGAT 120
134                AATAGTTGGTGTGCCGGCTATACCATGTCAGCACTACTCAATGCCACATATAATACAAAT 120
                   *** ********** ** ***** ********* **** ***** ** ********* **

24                 CGTTATAATGCTGAGGCAGTAATGAGATATTTACATCCGAATTTACAAGGTGATGATTTC 180
31179              CGTTATAATGCTGAGGCAGTAATGAGATATTTACATCCGAATTTACAAGGTGATGATTTC 180
134                CGATATAATGCAGAATCAGTAATGAGATATTTACATCCTAATTTAAGAGGTCACGACTTC 180
                   ** ******** **  ********************** ******  **** * ** ***

24                 CAATTTACAGGTTTAACACCTCAAGAAATGATGAAGTATGGGAAATCACAGGGTAGAGAT 240
31179              CAATTTACAGGTTTAACACCTCAAGAAATGATGAAGTATGGGAAATCACAGGGTAGAGAT 240
134                CAATTTACAGGACTAACATCTAACGAGATGCTTCGTTTTGGTAGATCACAAGGCAGAAAT 240
                   ***********  ***** ** * ** *** *    * *** * ****** ** *** **

24                 ACTCAATATCTTAATAGAATGCCATCTTATAATGAAGTTGATAAATTGACTACTAATAAT 300
31179              ACTCAATATCTTAATAGAATGCCATCTTATAATGAAGTTGATAAATTGACTACTAATAAT 300
134                ACTCAATATCTTAATAGAATGACTTCATATAATGAAGTAGACCAATTAACAACTAATAAT 300
                   ********************** * ** *********** **  **** ** *********

24                 AAAGGCATTGCTATTTTAGGAAGTCGTGTAGAATCAACAGACGGTATTCATGCAGGACAT 360
31179              AAAGGCATTGCTATTTTAGGAAGTCGTGTAGAATCAACAGACGGTATTCATGCAGGACAT 360
134                CAAGGTATAGCTGTATTAGGTAAGCGTGTTGAATCAAGCGATGGTATTCACGCTGGACAT 360
                    **** ** *** * ***** *  ***** *******  ** ******** ** ******

24                 GCTATGGCTGTAGTTGGTAATGCAGAATTAGAAGGTGGACAAGAAGTGATTATGATTTGG 420
31179              GCTATGGCTGTAGTTGGTAATGCAGAATTAGAAGGTGGACAAGAAGTGATTATGATTTGG 420
134                GCCATGGCTGTGGCTGGTAATGCTAAAGTTAACAACGGACAAAAAGTCATTTTAATTTGG 420
                   ** ******** * *********  ** *  *    ****** **** *** * ******

24                 AACCCTTGGGACCGTGGTTTTATGACTCAAGATGCTGAAAGTAATATTATTCCAGTATCT 480
31179              AACCCTTGGGACCGTGGTTTTATGACTCAAGATGCTGAAAGTAATATTATTCCAGTATCT 480
134                AACCCATGGGACAATGGTCTCATGACTCAAGATGCACATAGTAATATCATTCCAGTATCA 480
                   ***** ******  **** * **************  * ******** ***********

24                 AATGGAGATCACTATGAATGGTATGCATCAATTTATGGTTAT 522
31179              AATGGAGATCACTATGAATGGTATGCATCAATTTATGGTTAT 522
134                AATGGCGATCACTATGAATGGTATGCATCAATTTATGGTTAT 522
                   ***** ************************************
```

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[a]: Typical avian *S. aureus* isolates; [b]: Human *S. epidermidis* isolate.

b) Comparison of sequences of the coagulase gene region

```
[a]24            TAACGACAAATCAAGATGGCACAGTATCATACGGAGCTCGCCCAACACAAAACAAGCCAA 60
[a]85            TAACGACAAATCAAGATGGCACAGTATCATACGGAGCTCGCCCAACACAAAACAAGCCAA 60
[b]82            ------------------------------------------------------------
[c]RN4220        ------------------------------------------------------------
[b]496           ------------------------------------------------------------

24               GTGAAACAAACGCATATAACGTAACAACACATGCAAATGGTCAAGTATCATACGGTGCTC 120
85               GTGAAACAAACGCATATAACGTAACAACACATGCAAATGGTCAAGTATCATACGGTGCTC 120
82               ---------------ACAACGTAACAACACATGCAAATGGTCAAGTATCATATGGCGCTC 45
RN4220           ---------------ATAACGTAACAACACATGCAAATGGTCAAGTATCATACGGAGCTC 45
496              --------------TACAACGTAACGACAAATCAAGATGGCACAGTAACATATGGCGCTC 46
                             * ******** *** **  * ****   **** **** ** ****

24               GCCCAACACAAAACAAGCCAAGCAAAACAAATGCATACAACGTAACAACACATGCAAATG 180
85               GCCCAACACAAAACAAGCCAAGCAAAACAAATGCATACAACGTAACAACACATGCAAATG 180
82               GCCCGACACAAAACAAGCCAAGCAAAACAAATGCATATAACGTAACAACACATGCAAATG 105
RN4220           GTCCGACATACAAGAAGCCAAGCGAAACGAATGCATACAATGTAACAACACATGCAAACG 105
496              GCCCAACACAAAACAAACCAAGCAAAACAAATGCATACAACGTAACAACACATGCAAATG 106
                 * ** *** * ** ** ****** **** ******** ** ***************** *

24               GTCAAGTATCATATGGCGCTCGCCCGACACAAAAAAAGCCAAGCAAAACAAATGCATATA 240
85               GTCAAGTATCATATGGCGCTCGCCCGACACAAAAAAAGCCAAGCAAAACAAATGCATATA 240
82               GTCAAGTATCATACGGAGCTCGCCCGACATACAAGAAGCCAAGCGAAACAAATGCATACA 165
RN4220           GCCAAGTATCATACGGAGCTCGTCCGACACAAAACAAGCCAAGCAAAACAAACGCATATA 165
496              GTCAAGTATCATATGGCGCTCGCCCGACACAAAACAAGCCAAGCAAAACAAATGCATATA 166
                 * ************ ** ***** ****** * ** ********* ******* ***** *

24               ACGTAACAACACATGCAAATGGTCAAGTATCATACGGAGCTCGCCCGACATACAAGAAGC 300
85               ACGTAACAACACATGCAAATGGTCAAGTATCATACGGAGCTCGCCCGACATACAAGAAGC 300
82               ACGTAACAACACATGCAAATGGTCAAGTATCATATGGCGCTCGCCCGACACAAAACAAGC 225
RN4220           ACGTAACAACACATGGAAACGGCCAAGTATCATATGGCGCTCGCCCAACACAAAACAAGC 225
496              ACGTAACAACACATGCAAATGGTCAAGTATCATACGGAGCTCGCCCGACACAAAACAAGC 226
                 *************** *** ** ************ ** ******** *** * ** ****

24               CAAGCGAAACAAATGCATACAACGTAACAACACATGCAAATGGTCAAGTATCATATGGCG 360
85               CAAGCGAAACAAATGCATACAACGTAACAACACATGCAAATGGTCAAGTATCATATGGCG 360
82               CAAGCGAAACAAACGCATATAACGTAACAACACATGCAAACGGTCAAGTATCATACGGCG 285
RN4220           CAAGCAAAACAAATGCATACAACGTAACAACACATGCAAACGGTCAAGTGTCATACGGAG 285
496              CAAGCAAAACAAATGCATATAACGTAACAACACACGCAAACGGTCAAGTGTCATACGGAG 286
                 ***** ******** ***** ************** ***** ******** ***** ** *

24               CTCGCCCGACACAAAAAAAGCCAAGCGAAACAAACGCATATAACGTAACAACACATGCAG 420
85               CTCGCCCGACACAAAAAAAGCCAAGCGAAACAAACGCATATAACGTAACAACACATGCAG 420
82               CCCGCCCAACATACAAGAAGCCAAGCGAAACGAATGCATATAACGTAACAACACATGCAG 345
RN4220           CTCGCCCGACATACAAGAAGCCAAGTAAAACAAATGCATACAATGTAACAACACATGCAG 345
496              CTCGCCCGACATACAAGAAGCCAAGTAAAACAAATGCATACAATGTAACAACACATGCAG 346
                 * ***** *** * ** ********  **** ** ***** ** ****************

24               ATGGTACTGCGACATATGGGCCTAGAGTAACAAAATAAATTTATAACTCTATCCAAAGAC 480
85               ATGGTACTGCGACATATGGGCCTAGAGTAACAAAATAAATTTATAACTCTATCCAAAGAC 480
82               ATGGTACTGCGACATATGGGCCTAGAGTAACAAAATAAGTTTGTAACTCTATCCAAAGAC 405
RN4220           ATGGTACTGCGACATATGGGCCTAGAGTAACAAAATAAGTTTGTAACTCTATCCAAAGAC 405
496              ATGGTACTGCGACATATGGGCCTAGAGTAACAAAATAAGTTTGTAACTCTATCCAAAGAC 406
                 ************************************ *** *****************

24               ATACAGTCAATACAAAGAATTATGTATCTATACAACAGTAATCATGCATTCTATGATGCT 540
85               ATACAGTCAATACAAAGAATTATGTATCTATACAACAGTAATCATGCATTCTATGATGCT 540
82               ATACAGTCAATACAAAACATTACGTATCTTTACAACAGTAATCATGCATTCTATGATGCT 465
RN4220           ATACAGTCAATACAAAACATTACGTATCTTTACAACAGTAATCATGCATTCTATGATGCT 465
496              ATACAGTCAATACAAAACATTACGTATCTTTACAACAGTAATCATGCATTCTATGATGCT 466
                 ****************  **** ****** ******************************

24               TCTAACTGAATTAAAGCATCGAACAATCGGAAGCATATTTCTAAATTATTTATTCATTAT 600
85               TCTAACTGAATTAAAGCATCGAACAATCGGAAGCATATTTCTAAATTATTTATTCATTAT 600
82               TCTAACTGAATTAAAGCATCGAACAATCGGAAGCATATTTCTAAATTATTTATTCATTAT 525
RN4220           TCTAACTGAATTAAAGCATCGAACAATCGGAAGCATATTTCTAAATTATTTATTCATTAT 525
496              TCTAACTGAATTAAAGCATCGAACAATCGGAAGCATATTTCTAAATTATTTATTCATTAT 526
                 ************************************************************

24               AGTCTTAAACATAACATGACCTAATATATTACTAACCTATTAAAATAAACCACGCACATC 660
85               AGTCTTAAACATAACATGACCTAATATATTACTAACCTATTAAAATAAACCACGCACATC 660
82               AGTCTTAAACATAACATGACCTAATATATTACTAACCTATTAAAATAAACCACGCACATC 585
RN4220           AGTCTTAAACATAACATGACCTAATATATTACTAACCTATTAAAATAAACCACGCACATC 585
496              AGTCTTAAACATAACATGACCTAATATATTACTAACCTATTAAAATAAACCACGCACATC 586
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b) Continued ...

b) Continued ...

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24              TAAGTGATATACGACAATCACAGCAATAATAATTGCTTTAGAAAGTCGTACCGAACTGGA 720
85              TAAGTGATATACGACAATCACAGCAATAATAATTGCTTTAGAAAGTCGTACCGAACTGGA 720
82              TAAGTGATATACGACAATCACAGCAATAATAATTGTTTTAGAAAGTCGTACCGAACTGGA 645
RN4220          TAAGTGATATACGACAATCACAGCAATAATAATTGCTTTAGAAAGTCGTGCCGAACTGGA 645
496             TAAGTGATATACGACAATCACAGCAATAATAATTGCTTTAGAAAGTCGTGCCGAACTGGA 646
                ********************************** ************ **********

24              ACTTACAAGTCTAGTTCGAACACACACTGATGTGAGTGG 759
85              ACTTACAAGTCTAGTTCGAACACACACTGATGTGAGTGG 759
82              ACTTACAAGTCTAGTTCGAACACACACTGATGTGAGTGG 684
RN4220          ACTTACAAGTCTAGTTCGAACACACACTGATGTGAGTGG 684
496             ACTTACAAGTCTAGTTCGAACACACACTGATGTGAGTGG 685
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[a]: Typical avian *S. aureus* isolates; [b]: Atypical avian *S. aureus* isolates; [c]: Human *S. aureus* isolate.

c) Comparison of sequences of the *agrBDC* region

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[a]82              ---------TATTAAACGAAAAAAATATTATGCGATTATTGTTAGTTTAACCCTTTTCAT 51
[b]RN4220          --------TTATTAAACGAAAAAAATATTATGCGATTATTGTTAGTTTAACCCTTTTCAT 52
[a]496            AGCGAGGCTTGTAAAGCAAAAAAGATATTTTTCAATTATAATTAGTACAATTCTTTTCAT 60
[c]24              --TAAAATTAGTAAAACGTAAAAAGTATTTATCTATAATTATGTATTTATTAGTTTTGAT 58
[c]85              --TAAAATTAGTAAAACGTAAAAAGTATTTATCTATAATTATGTATTTATTAGTTTTGAT 58
                             * ** *  ****  ****   * ** **  *   *  *    **** **

82                 TATCACACTTATCATCAAAGAGCCATTTGCCCAATTCATTCAATTAGGCATCATAATAGA 111
RN4220             TATCACACTTATCATCAAAGAGCCATTTGCCCAATTCATTCAATTAGGCATCATAATAGA 112
496                TATCACACTTTTTGTAAAAGAACCATATACCCAATTTATTCAACTGGGTATTATTATACA 120
24                 TCTATCCTTAATAATACATCCATTTTATGCTCAATTTATGTTATTAGGAATTTTAGTAGA 118
85                 TCTATCCTTAATAATACATCCATTTTATGCTCAATTTATGTTATTAGGAATTTTAGTAGA 118
                   * *  *  *  *  *  *       * * * ***** **   * * ** **  *  ** *

82                 AGCTATTACATTATTACCTATTTTCTTTATTAAGGAGGACTTAAA-ATGAATACATTATT 170
RN4220             AGCTATTACATTATTACCTATTTTCTTTATTAAGGAGGACTTAAA-ATGAATACATTATT 171
496                AGCTATTACATTACTACCAATATACTATTCAAAGGAGGACTAAATTATGAAAAAATTACT 180
24                 ATCTATTACATTATTACCTATTTTTTTCCCTAAGGAGGA-TTAATCATGAATACACTTGT 177
85                 ATCTATTACATTATTACCTATTTTTTTCCCTAAGGAGGA-TTAATCATGAATACACTTGT 177
                   * ********** **** ** *  *     ******** * **  ***** * * *  *

82                 TAACTTATTTTTTGATTTTATTACTGGGATTTTAAAAAACATTGGTAACATCGCAGCTTA 230
RN4220             TAACTTATTTTTTGATTTTATTACTGGGATTTTAAAAAACATTGGTAACATCGCAGCTTA 231
496                CAACAAAGTTATTGAGCTTTTAGTTGACTTTTTCAACAGCATCGGTTACAGAGCCGCGTA 240
24                 TAATATGTTTTTTGATTTTATAATTAAATTAGCTAAAGCAATCGGAATTGTCGGTGGCGT 237
85                 TAATATGTTTTTTGATTTTATAATTAAATTAGCTAAAGCAATCGGAATTGTCGGTGGCGT 237
                    **     ** ****  ** *   *    *    **    ** **       *  *

82                 TAGTACTTGTGACTTCATAATGGATGAAGTTGAAGTACCAAAAGAATTAACACAATTACA 290
RN4220             TAGTACTTGTGACTTCATAATGGATGAAGTTGAAGTACCAAAAGAATTAACACAATTACA 291
496                TATAAATTGTGATTTTTTATTGGACGAAGCTGAAGTACCAAAAGAATTAACTCAATTACA 300
24                 AAACGCTTGCAGCAGTTTATTTGATGAACCTAAAGTACCCGCTGAATTAACGAATTTATA 297
85                 AAACGCTTGCAGCAGTTTATTTGATGAACCTAAAGTACCCGCTGAATTAACGAATTTATA 297
                    *    ***         ** * ** ***  * *******    ********  * *** *

82                 CGAATAATTTAAATAGAGAGTGT-GATAGTAGGTGGAATTATTAAATAGTTA-TAATTTT 348
RN4220             CGAATAATTTAAATAGAGAGTGT-GATAGTAGGTGGAATTATTAAATAGTTA-TAATTTT 349
496                CGAATAACTATAAGAGAAAGTGT-GATAGTAAGTGGAAGCATTAAATGATTA-TAATTAC 358
24                 CGACAAATAGTAAAAGTTATTTTAGAAAGTGTGTAGCATATGGAAACAATAAACAACTTA 357
85                 CGACAAATAGTAAAAGTTATTTTAGAAAGTGTGTAGCATATGGAAACAATAAACAACTTA 357
                   ***  **    ** **  * * * ** ***  ** * *     ***   * *  ** *

82                 GTTTTATTCGTATTAACTCAAATGATATTAATGTTTACAATACCAGCTATAATTAGTGGT 408
RN4220             GTTTTATTCGTATTAACTCAAATGATATTAATGTTTACAATACCAGCTATAATTAGTGGT 409
496                GTTTTATTTGTAATTGTTCAAGTTTCATTAATGTTCTTTATATCTGCATTTATTAGTGGA 418
24                 GCTATGGCCACTTT---TCAATTAGTAATACTTTTTACAGTAGCCAAATTCATTTCCTTT 414
85                 GCTATGGCCACTTT---TCAATTAGTAATACTTTTTACAGTAGCCAAATTCATTTCCTTT 414
                   * * *        *   **** *   * ** * **     ** *     * ***

82                 ATTAAGTACAGTAAACTTGATTATTTTTTCATTATAGGAATTTCGACATTATCGTTATTT 468
RN4220             ATTAAGTACAGTAAACTTGATTATTTTTTCATCATAGTAATTTCGACATTATCGTTATTT 469
496                ATAAGATACAAAAAATCAGATTATATATACATTATTGGAATAGTGTTATCTTCAGTATAT 478
24                 GTTAAATTTAATTTAAGGGATTATTTTATTATAGTTGGAATTATAATTCCAACCATGTTT 474
85                 GTTAAATTTAATTTAAGGGATTATTTTATTATAGTTGGAATTATAATTCCAACCATGTTT 474
                    * *  *  *    *   ****** *    **  * * ***          *  * * *

82                 CTATTTAAAATGTTTGATAGCGCGTCCTTAATCATTTTAACTTCATTCATTATTATAATG 528
RN4220             CTATTTAAAATGTTTGATAGCGCGTCCTTAATCATATTAACTTCATTTATTATTATAATG 529
496                TTCTTTGACAAAATCGGAAGTATTTCACTAGTTGTTATAACAATTTTTATAATAATTTTT 538
24                 TTGTATTACTTCTATGGAAGTAGAGCCGTATTGATTCCAACTTTTTCATCTATCATATTT 534
85                 TTGTATTACTTCTATGGAAGTAGAGCCGTATTGATTCCAACTTTTTCATCTATCATATTT 534
                    * * * *       *  **     *  ** *  *   ***    *     ** **  *

82                 TATTTTGTCAAAATCAAATGGTATTCTATTTTGTTGATTATGACTTCACAGATTATTCTG 588
RN4220             TATTTTGTCAAAATCAAATGGTATTCTATTTTGTTGATTATGACTTCGCAGATTATTCTA 589
496                CTCTATTTTAAAATTAGACTATATTCTGTATTTTTAGTAATGGTTACTCAAATCATTTTA 598
24                 TTATTCTTTAAATTAAAGTACTATGCAATAGTAACAATTTTAGTGACCATGATCATAATG 594
85                 TTATTCTTTAAATTAAAGTACTATGCAATAGTAACAATTTTAGTGACCATGATCATAATG 594
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c) Continued …

c) Continued ...

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82          TACTGTGCTAACTACATGTATATTGTTATATATGCATATATCACCAAGATTTCTGATAGT 648
RN4220      TACTGTGCTAACTACATGTATATAGTTATATATGCATATATCACCAAAATTTCTGATAGT 649
496         TATTGTGCAAATTTCGTTTATATCATTATTTTTTCATACATTATAACAATTTCACACAGC 658
24          TATTTGAGTAATTTTGCAACAGTAGGTTTGTTTTTAACTTTAAGAAAATACACTACTGAT 654
85          TATTTGAGTAATTTTGCAACAGTAGGTTTGTTTTTAACTTTAAGAAAATACACTACTGAT 654
            ** *     ** *          *   * * * * *    * * *      *

82          ATATTTGTAATATTCCCTAGCTTTTTTGTAGTTTATGTGACTATTAGTATACTATTCT-C 707
RN4220      ATATTTGTAATATTCCCTAGCTTTTTTGTAGTTTATGTGACTATTAGTATACTATTCT-C 708
496         GTGTTTATAGTTCTACCAATCTTTTTGGTAGTGTATGTATCAATTTCTTATGCACTTG-C 717
24          CCTGCAATACTCTTACCATTATATATACTTTCTTTTTCATCAGTTTCATTATTAGCCGAC 714
85          CCTGCAATACTCTTACCATTATATATACTTTCTTTTTCATCAGTTTCATTATTAGC-GAC 713
                   ** *  * **    * * *  *    * *    *  **        *    *

82          ATATATAATAAATAGAGTTCTCAAAAAAATTAGCACACCATATCTAATACTAAACAAAGG 767
RN4220      ATATATAATAAATAGAGTTCTCAAAAAAATTAGCACACCATATCTAATACTAAACAAAGG 768
496         CTACATTTTAAATAGAATATTAAAAAGGATAAATGGTACATACCTTTCTCTTAATAAAAA 777
24          ATATTTGGTAAGAATATCTCTAAAGAAATTTAAAAAATCATACTTATCTTTAAATAAAAC 774
85          ATATTTGGTAAGAATATCTCTAAAGAAATTTAAAAAATCATACTTATCTTTAAATAAAAC 773
             **  *  ***  * *    * ** *  * *      ****  *     * ** ***

82          ATTTTTAATAGTTATTTCGACTATCTTACTGCTCACTTTTTCATTATTTTTCTTTTATTC 827
RN4220      ATTTTTAATAGTTATTTCGACTATCTTACTGCTTACTTTTTCATTATTTTTCTTTTATTC 828
496         ATTCCTTACTGTTATAACTATTGTTATAGTAATAACATTCTCTTTGTTATTTGCATATTC 837
24          TTATATGATTATAATTTCATTCGTATTATTTGCAACATTTGCATTTTTTTATATTTATTC 834
85          TTATATGATTATAATTTCATTCGTATTATTTGCAACATTTGCATTTTTTTATATTTATTC 833
             *   * *   * **  *     *  ** *    ** **  * ** ** *     *****

82          -ACAAATA--AACTCGGATGAAGCTAAAGTAATAAGGCAGTATTCTTTTATTTTTATTGG 884
RN4220      -ACAAATA--AACTCGGATGAAGCTAAAGTAATAAGGCAGTATTCTTTTATTTTTATTGG 885
496         -GCAAATT--GATGCGAGTGATGCATCAACAATCAAACAGTATTCGCTTTTATTCTTGGG 894
24          TACAAATACAAGTTCAAACGGTGATAGCTTAATT---CCATATGCACTTGTATTTATAGG 891
85          TACAAATACAAGTTCAAACGGTGATAGCTTAATT---CCATATGCACTTGTATTTATAGG 890
              *****       *    *  *       ***    *  *** *  ** * **  * **

82          TATCACTATATTTTTAAGTATATTAACATTTGTTATTTCTCAATTTCTCCTTAAAGAGAT 944
RN4220      TATCACTATATTTTTAAGTATATTAACATTTGTTATTTCTCAATTTCTCCTTAAAGAGAT 945
496         AATTATTATATTACTGAGTATCTTAATTTTTATTTATTCTCAATTTACTCTTAAAGAAAT 954
24          ATTAATAATTTTTATCTCTGTTGTAATACTTATAATGTCGCTTTTCACCCTTAAAGAGAT 951
85          ATTAATAATTTTTATCTCTGTTGTAATACTTATAATGTCGCTTTTCACCCTTAAAGAGAT 950
              * *  ** **  *   * *  ***   ** *    ** *  **    ******** **

82          GAAATATAAACGTAATCAAGAAGAAATTGAAACCTATTA---------------- 983
RN4220      GAAATATAAACGTAATCAAGAAGAAATTGAAACCTATTA-GAATATAC------ 992
496         GAAATATAAACGTAATCAAGAAGAAATTGAAACCTATTATGAATACACATTAAA 1008
24          GAAATACAAACGAAATCAAGAAGAAATTGAGACCTATTACGAATACACATTAAA 1005
85          GAAATACAAACGAAATCAAGAAGAAATTGAGACCTATTACGAATACACATTAAA 1004
            ****** ***** ***************** ********
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[a]: Atypical avian *S. aureus* isolates; [b]:Human *S. aureus* isolate; [c]: Typical avian *S. aureus* isolates.

d) Comparison of sequences of the *agrD* gene

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[a]24              ATGAATACACTTGTTAATATGTTTTTTGATTTTATAATTAAATTAGCTAAAGCAATCGGA 60
[a]85              ATGAATACACTTGTTAATATGTTTTTTGATTTTATAATTAAATTAGCTAAAGCAATCGGA 60
[b]82              ATGAATACATTATTTAACTTATTTTTTGATTTTATTACTGGGATTTTAAAAAACATTGGT 60
[c]RN4220          ATGAATACATTATTTAACTTATTTTTTGATTTTATTACTGGGATTTTAAAAAACATTGGT 60
[d]496             ATGAAAAAATTACTCAACAAAGTTATTGAGCTTTTAGTTGACTTTTTCAACAGCATCGGT 60
                   ***** * * *  * **     ** ****  ** *   *    *    **    ** **

24                 ATTGTCGGTGGCGTAAAC-GCTTGCAGCAGTTTATTTGATGAACCTAAAGTACCCGCTGA 119
85                 ATTGTCGGTGGCGTAAAC-GCTTGCAGCAGTTTATTTGATGAACCTAAAGTACCCGCTGA 119
82                 AACAT-CGCAGCTTATAGTACTTGTGACTTCATAATGGATGAAGTTGAAGTACCAAAAGA 119
RN4220             AACAT-CGCAGCTTATAGTACTTGTGACTTCATAATGGATGAAGTTGAAGTACCAAAAGA 119
496                TACAG-AGCCGCGTATATAAATTGTGATTTTTTATTGGACGAAGCTGAAGTACCAAAAGA 119
                         *  ** ** *    ***        ** * ** ***  * *******    **

24                 ATTAACGAATTTATACGACAAATAG 144
85                 ATTAACGAATTTATACGACAAATAG 144
82                 ATTAACACAATTACACGAATAA--- 141
RN4220             ATTAACACAATTACACGAATAA--- 141
496                ATTAACTCAATTACACGAATAA--- 141
                   ******  * *** ****  **
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[a]: Typical avian *S. aureus* isolates (group II of the autoinducing peptides);
[b]: Atypical avian *S. aureus* isolate (group I of the autoinducing peptides);
[c]:Human *S. aureus* isolate (group I of the autoinducing peptides);
[d]: Atypical avian *S. aureus* isolate (group III of the autoinducing peptides).

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