A STUDY OF THE MOLECULAR DIVERSITY OF *STAPHYLOCOCCUS AUREUS* PLASMIDS AND THEIR ROLE IN THE CHARACTERISATION OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

BY

JOHN E. COIA B.Sc. M.B. Ch.B.

being a thesis submitted for the Degree of Doctor of Medicine in the University of Glasgow, Faculty of Medicine, October 1990

To Bernadette, Wee John and Mum, who are simply the best, and deserve the real credit

TABLE OF CONTENTS

TITLE PAGE 1

TABLE OF CONTENTS 3

LIST OF TABLES 7

LIST OF ILLUSTRATIONS 9

ACKNOWLEDGEMENTS 11

DECLARATION 12

SUMMARY 13

ABBREVIATIONS 18

1 INTRODUCTION 23

MRSA in Scotland 28
Mechanism of methicillin-resistance 29
Epidemiology of MRSA infection 31
Traditional Methods 32
Recent developments 34
Proteins 35
Nucleic acids 37
Plasmid profiles 37
Restriction enzyme fragmentation patterns 40
Chromosomal REFPs 43
DNA probes 44
Pathogenicity of *Staphylococcus aureus* 47
Cell wall associated factors 47
Extracellular products 48
Enzymes 48
Toxins 50
Pathogenicity of MRSA 53

2 MATERIALS AND METHODS 57

Bacterial Strains 58
Methicillin-sensitive *Staphylococcus aureus* (MSSA) 58
Methicillin-resistant *Staphylococcus aureus* (MRSA) 58
Isolation and identification of organisms 59
Antibiotic susceptibility testing 59
Phage typing 60
Biotyping 60
Preparation of immunoblots of exported proteins 61
Determination of plasmid profiles 63
Preparation of purified plasmid DNA 64
Restriction endonuclease digestion 66
Enterotoxin production 67
Detection of Haemolysins 68
α-haemolysin 69

β-haemolysin 70
γ-haemolysin 70
δ-haemolysin 70
Statistical analysis 71

3 RESULTS 72

Antibiotic susceptibility testing 73
Antibiograms 73
Aminoglycoside-resistance 74
Mupirocin-resistance 74
Biotyping 75
Immunoblotting 76
Plasmid profiles 77
Plasmid-bearing isolates 77
Distribution of plasmids 77
Plasmid sizes 78
Number of REFPs 79
MRSA isolates 79
MRSA(1) 79
MRSA(2) 81
MSSA isolates 82
Common features 82
Small plasmids 83
Size heterogeneity 83
Comparison of biotyping, immunoblotting, REFPs and phage typing of MRSA(1) 84
Pathogenicity factors 85
Enterotoxin production 85
Distribution of individual enterotoxins 86
Enterotoxin A 86
Enterotoxin B 86
Enterotoxin C 87
Enterotoxin D 87
Distribution of enterotoxin-producing strains 87
Haemolysin Production 89
Distribution of individual haemolysins 90
α- haemolysin 90
β- haemolysin 91
γ- haemolysin 91
δ- haemolysin 92
Distribution of haemolysin-producing strains 92

4 DISCUSSION 94

Antibiotic resistance 95
Prevalence and distribution of plasmids in MSSA AND MRSA 95
Comparison of MSSA and MRSA REFPs 97
Origins of MRSA within GRI 100
Comparison of characterisation of MRSA by biotyping, immunoblotting and REFPS 102
Pathogenicity factors 108

Enterotoxin production 108
MSSA isolates 109
MRSA isolates 111
MRSA and MSSA compared 113
Haemolysins 114
MSSA 115
MRSA 116
MRSA and MSSA compared 117
General considerations 117
Future developments 119

5 CONCLUSIONS 126

APPENDIX 1 Origins, antibiotic sensitivities, number of plasmids harboured and REFPs of *Staph. aureus* isolates 129

APPENDIX 2 Phage-types of MRSA 154

APPENDIX 3 Enterotoxin and haemolysin production by *Staph. aureus* from different sources 157

REFERENCES 175

LIST OF TABLES

Table I. Penicillin binding proteins (PBPs) of *Staphylococcus aureus* 30
Table II. Examples of palindromic restriction endonuclease enzyme cleavage sites 41
Table III. Extracellular enzymes of *Staphylococcus aureus* 49
Table IV. Toxins of *Staphylococcus aureus* 50
Table V. Numbers of isolates in each collection resistant to individual antibiotics 73
Table VI. Distribution of resistance determinants in isolates from different sources 74
Table VII. Distribution of MRSA antibiograms 75
Table VIII. Biotypes of MRSA 76
Table IX. Frequency distribution of plasmids in isolates from different sources 77
Table X. Frequency distribution of plasmids in combined MSSA and MRSA isolates compared 78
Table XI. REFP group, immunoblot group, biotype and phage type (RTD) of MRSA(1) 84
Table XII. Distribution of enterotoxin production in isolates from different sources 85
Table XIII. Comparison of numbers of enterotoxin producers and non-producers amongst isolates from different sources 88
Table XIV. Comparison of multiple enterotoxin production by isolates from different sources 88
Table XV. Distribution of haemolysin production in isolates from different sources 90
Table XVI. Comparison of numbers of haemolysin producers and non-producers amongst isolates from different sources 92
Table XVII. Comparison of multiple haemolysin production by isolates from different sources 93
Table XVIII. Comparison of observed plasmid distributions in MSSA and MRSA with distributions predicted by a modified Poisson model 97
Table XIX. Distribution of enterotoxin production in isolates from different sources - Dublin and Glasgow compared 110
Table XX. Distribution of haemolysin production in isolates from different sources - Dublin and Glasgow compared 116

LIST OF ILLUSTRATIONS

Figure 1. Structure of methicillin and the isoxazolyl penicillins, cloxacillin and flucloxacillin....26
Figure 2. Comparison of the distribution of resistance determinants/isolate of *Staph. aureus* from different sources....73
Figure 3. Typical immunoblots of MRSA culture supernates....76
Figure 4. Frequency distributions of plasmids in isolates from different sources....78
Figure 5. Comparison of frequency distributions of plasmids in MSSA and MRSA isolates....78
Figure 6. Typical *S. aureus* plasmid profiles....79
Figure 7. Typical *S. aureus* plasmid profiles....79
Figure 8. Typical *Hae*III restriction patterns of *S. aureus*....79
Figure 9. Typical *Hae*III restriction patterns of MRSA....79
Figure 10. *Hae*III restriction patterns of MRSA(1)....79
Figure 11. *Hae*III restriction patterns of MRSA(2)....81
Figure 12. Examples of *Hae*III restriction patterns of *S. aureus*....82
Figure 13. Typical *Hae*III restriction patterns of MRSA....82
Figure 14a. *Alu*I restriction patterns of *S. aureus*....83
Figure 14b. *Alu*I restriction patterns of *S. aureus*....83
Figure 15. Correlation of immunoblot, REFP group and biotype of MRSA(1) isolates....84
Figure 16. Results of RPLA tests for enterotoxins of *Staph. aureus*....85
Figure 17. Distribution of multiple enterotoxin production by *Staph. aureus* from different sources....89
Figure 18. Results of tests for haemolysins of *Staph. aureus*....89
Figure 19. Distribution of multiple haemolysin production by *Staph. aureus* from different sources....93
Figure 20. Restriction enzyme fragmentation patterns of two hypothetical related plasmids (a) and (b)....122
Figure 21. *Pst*I chromosomal fingerprints of *Staph. aureus*....124
Figure 22a *Hae*III restriction enzyme fragmentation patterns of *Staph. aureus*; patterns (1-19)....130
Figure 22b *Hae*III restriction enzyme fragmentation patterns of *Staph. aureus*; patterns (20-38)....130
Figure 22c. *Hae*III restriction enzyme fragmentation patterns of *Staph. aureus*; patterns (39-56)....130

ACKNOWLEDGEMENTS

I would like to thank Professor Soad Tabaqchali and Dr Barry Cookson for providing EMRSA isolates, and Dr Brian Keighley for assistance in collecting community isolates. Special thanks to Drs Dugald Baird, Harry Birkbeck, David Platt and Morag Timbury for all their encouragement and assistance. Particular thanks to Professor Douglas Sleigh for all his advice and encouragement in the preparation of this work.

Final thanks to Sheila and Joseph Lynch for helping to check this manuscript.

DECLARATION

The experiments and composition of this thesis are the work of the author unless otherwise stated.

Two hundred and sixty-four isolates of *Staphylococcus aureus* were collected; 163 methicillin-sensitive *Staph. aureus* (MSSA) and 101 methicillin-resistant *Staph. aureus* (MRSA). MSSA comprised: from Glasgow Royal Infirmary (GRI) 50 blood culture isolates (BC), 63 isolates from routine diagnostic specimens (RD) and fifty strains from nasal swabs of patients attending a general practitioner (GP). The MRSA from GRI comprised: 53 isolates from 1985-86 (MRSA(1)) and 48 isolates from 1986-87 (MRSA(2)).

The number, frequency distribution and diversity of plasmids harboured by these strains was compared by means of plasmid profiles and restriction enzyme fragmentation patterns (REFPs). Plasmids were demonstrated in less than half the MSSA; their frequency distribution did not differ from that predicted by a simple model of plasmid distributions. In contrast, all MRSA harboured plasmids and their distribution differed significantly from the MSSA.

Among 71 MSSA harbouring plasmids, 38 REFPs were identified. There were fewer REFPs among MRSA isolates; 11 MRSA(1) and 11 MRSA(2) patterns were observed, of which four were common to both collections. No MSSA patterns were identified amongst MRSA REFPs. The plasmid diversity of MRSA(1) and MRSA(2) isolates did not differ. Two predominant MRSA REFPs accounted for 70 % of those identified amongst MRSA and Dice analysis of all MRSA patterns defined two main

subgroups, FP1 and FP2, which correlated with the presence or absence of aminoglycoside-sensitivity.

Two size groups of plasmids, large and small could be discerned amongst *Staph. aureus* isolates. In the MRSA the large plasmids comprised two main species and molecular variants thereof; large MSSA plasmids were more diverse, and did not occur in common between MSSA and MRSA. Small plasmids were present only in MRSA and MSSA isolates which harboured more than one plasmid. Such plasmids were represented by a single multicopy species of 2.6 kb, which yielded the same REFP irrespective of its source.

Forty-five MRSA(1) isolates were further characterised by means of phage typing, simple biotyping and immunoblotting of exported proteins. The strains were subdivided into four groups (A-D) on the basis of biotype. Immunoblotting generated a number of unique patterns which were separated by Dice analysis into two major immunoblot groups (Blot1 and Blot2). Comparison of REFP and immunoblot groups revealed strong positive correlation between FP1 and Blot1 groups and between FP2 and Blot2 groups. In addition Blot1/FP1 isolates were almost exclusively of biotypes A or C, whereas Blot2/FP2 isolates were of biotypes B or D.

Comparison of production of enterotoxins A, B, C and D, and α–, β–, γ– and δ- haemolysins by MSSA and MRSA(1) isolates was performed by reverse passive latex agglutination (RPLA) and

agar overlay methods respectively. Sixty percent of BC MSSA produced enterotoxin; similar rates were found amongst other MSSA. Eighty-seven percent of aminoglycoside-sensitive MRSA produced enterotoxin; 89 % enterotoxin A alone. Only 27 % of aminoglycoside-resistant MRSA were enterotoxin-positive; significantly less than either MSSA or aminoglycoside-sensitive MRSA.

The proportion of haemolysin producing isolates did not differ amongst MSSA and MRSA; unlike enterotoxin production there was no difference in the distributions of haemolysins between aminoglycoside-sensitive and resistant strains of MRSA. GP MSSA had higher and lower numbers of γ- and δ- haemolysin producers respectively than other *Staph. aureus*. α- haemolysin producers were commoner amongst MRSA isolates, which also were more likely to produce multiple haemolysins than their methicillin-sensitive counterparts.

Information derived from plasmid distributions, REFP analysis, biotyping, immunoblot analysis and enterotoxin data were all compatible with a hypothesis of dissemination of a limited number of MRSA clones within GRI. The ubiquity of small plasmid species amongst MSSA and MRSA isolates raises the possibility that such clones may have arisen locally amongst endogenous methicillin-sensitive strains. The use of a combination of methods as described here has provided comprehensive epidemiological

information which has been valuable in studying the origin and spread of MRSA.

ABBREVIATIONS

AMBIS	Automated bacterial identification system
BC	Blood culture isolates of *Staph. aureus*
BIO A	MRSA biotype A
BIO B	MRSA biotype B
BIO C	MRSA biotype C
BIO D	MRSA biotype D
Blot1	MRSA immunoblot 1
Blot2	MRSA immunoblot 2
Blot3	MRSA immunoblot 3
C	Chloramphenicol
Cn	Gentamicin
CPHL	Central public health laboratory, Colindale
Da	Clindamycin
DNA	Deoxyribonucleic acid
EDTA	Ethylene-diamine-tetra-acetic acid
EMRSA	Epidemic methicillin-resistant *Staph. aureus*
Ery	Erythromycin
ETA	*Staph. aureus* epidermolytic toxin A
ETB	*Staph. aureus* epidermolytic toxin B
Fd	Fusidic Acid
FP1	MRSA restriction enzyme fragmentation pattern group 1

FP2	MRSA restriction enzyme fragmentation pattern group 2
FP3	MRSA restriction enzyme fragmentation pattern group 3
FP4	MRSA restriction enzyme fragmentation pattern group 4
GP	Community isolates of *Staph. aureus*
GRI	Glasgow Royal Infirmary
IgG	Immunoglobulin G
Km	Kanamycin
Met	Methicillin
MRSA	Methicillin-resistant *Staph. aureus*
MRSA(1)	MRSA isolates collected between February 1985 and April 1986
MRSA(2)	MRSA isolates collected between May 1986 and July 1987
MRSA(R)	Aminoglycoside-resistant MRSA
MRSA(S)	Aminoglycoside-sensitive MRSA
MSSA	Methicillin-sensitive *Staph. aureus*
Mup	Mupirocin
P	Penicillin
PBP	Penicillin binding protein
PBS	Phosphate-buffered saline
R_C	Critical radius of inhibition

RD	Routine diagnostic isolates of *Staph. aureus*
Rd	Rifampicin
rDNA	DNA encoding ribosomal genes
REFP	Restriction enzyme fragmentation pattern
RNA	Ribonucleic acid
RPLA	Reverse passive latex agglutination
rRNA	Ribosomal ribonucleic acid
RTD	Routine test dilution
S_D	Dice co-efficient of similarity
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SET-RPLA	Staphylococcal enterotoxin reverse passive latex agglutination test
Sm	Streptomycin
TE	a solution containing 10 mM Tris and 1 mM EDTA, pH 8.0
Tec	Teicoplanin
TES	a solution containing 50 mM Tris, 50 mM NaCl and 5 mM disodium EDTA, pH 8.0
TESS	TES containing 50 mM sucrose
Tet	Tetracycline

TSST-1	Toxic shock syndrome toxin 1
V/cm	Volts per centimetre
v/v	Volume per volume
Va	Vancomycin
W	Trimethoprim
w/v	Weight per volume

CHAPTER 1

Introduction

Forty years after the introduction of effective antimicrobial chemotherapy, pyogenic infections due to *Staphylococcus aureus* remain a significant cause of morbidity and mortality, both within the hospital environment and the community at large. The spectrum of disease produced by these organisms is very wide, ranging from superficial skin lesions to severe systemic infections (Shanson, 1986). The range of pathogenic processes which may be involved is almost uniquely wide, encompassing classical toxin mediated phenomena through direct tissue destruction to secondary immune mechanisms (Adlam and Easmon, 1983). The increasing numbers of nosocomial infections caused by *Staph. aureus*, especially those strains which are resistant to multiple antibiotics, will continue to present a major problem for all of those involved in the control and treatment of infection.

Staphylococci are non-motile, catalase-positive facultative anaerobes which grow in irregular clusters (Baird-Parker, 1972; Oeding, 1983). The demonstration of free coagulase and heat-stable deoxyribonuclease are the key tests for the differentiation of *Staph. aureus* from other staphylococci (Bergey's Manual, 1974). Hajek and Marsalek (1971) described six biotypes (A-F) of *Staph. aureus*, a classification supported by the results of Devriese and Oeding (1976); essentially those *Staph. aureus* isolates which are pathogenic for man belong to biotype (A) (Oeding, 1983).

The pathogenic potential of staphylococci has been recognised for over a century, since Sir Alexander Ogston first

described their causal role in the production of acute abscesses and septicaemia (Ogston, 1881). Nonetheless, in the pre-antibiotic era the prognosis for patients with severe staphylococcal disease was extremely poor. The introduction of penicillin into clinical use in the 1940s represented a dramatic breakthrough in the therapy of staphylococcal infections and transformed the previously dismal outlook for invasive and deep-seated disease (Plorde and Sherris, 1974). However, this triumph was short-lived; the history of the emergence of resistance in *Staph. aureus* provides a paradigm for the interaction between pathogenic microorganisms and antimicrobial agents. Penicillin-resistant ß-lactamase producing strains, initially described by Spink (Spink and Ferris, 1945), rapidly became widespread and by 1946 constituted some 60 % of hospital isolates of *Staph. aureus* in the United Kingdom (Barber, 1947; Barber and Rozwadoska-Dowzenko, 1948). The successive introduction of streptomycin, tetracycline, chloramphenicol, and the macrolides were all followed by the emergence of resistant organisms (Plorde and Sherris, 1974; Shanson, 1981). Most of the strains which were resistant to these new agents continued to synthesise a ß-lactamase, and the emergence of multiply-antibiotic-resistant *Staph. aureus* presented a significant health care problem during the 1950s. Amongst these strains were those belonging to the notorious phage type 80/81, which in addition to their multiple antibiotic resistance demonstrated particularly enhanced pathogenicity and communicability, often causing severe cutaneous

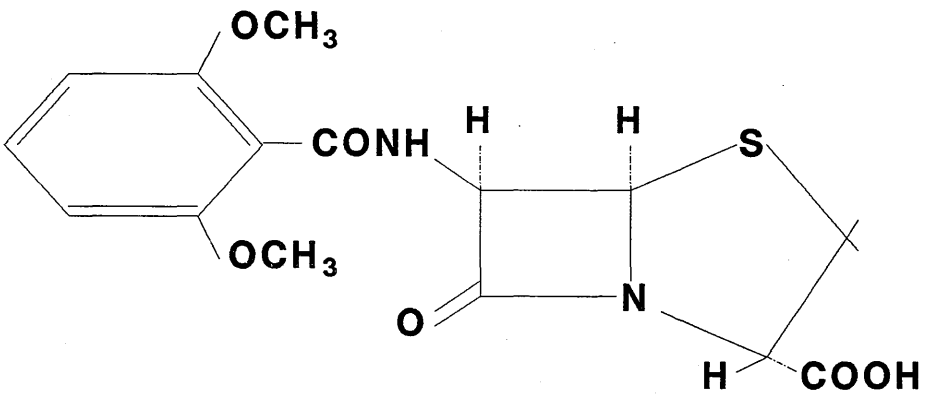
sepsis on the unbroken skin of attending medical and nursing staff (Rountree, 1978; Shanson, 1981; Parker, 1983).

The advent of methicillin, and the penicillinase-resistant semisynthetic isoxazolyl penicillins, once again provided the means with which to counter the rising tide of nosocomial staphylococcal infection (Knudsen and Rolinson, 1960; Knox, 1960). Due to the renal toxicity of methicillin (Baldwin *et al*, 1968) and the requirement for parenteral administration, it was quickly superseded in clinical practice by other similar drugs, particularly cloxacillin and flucloxacillin (Figure 1) which remain to this day the mainstay of therapy for *Staph. aureus* infections.

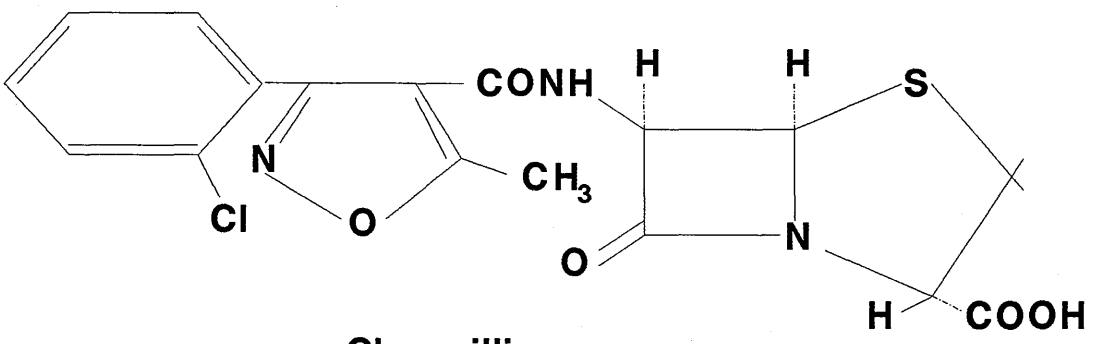
However, within months of the introduction of methicillin into clinical practice, methicillin-resistant[1] strains of *Staphylococcus aureus* (MRSA) were reported in Britain (Barber, 1961; Jevons, 1961). In the subsequent 10 years these strains became quite widespread, particularly in some hospitals in the UK and Europe. (Parker and Hewitt, 1970; Kayser and Mak, 1972). Prevalences of the organisms varied between 1-2 % in some countries to 20 % and above in others. However, their prevalence declined in the early 1970s for reasons which remain largely unexplained (Jepsen, 1986). For example between 1971 and 1975 the incidence of staphylococcal infections caused by MRSA in Zurich hospitals decreased from

---

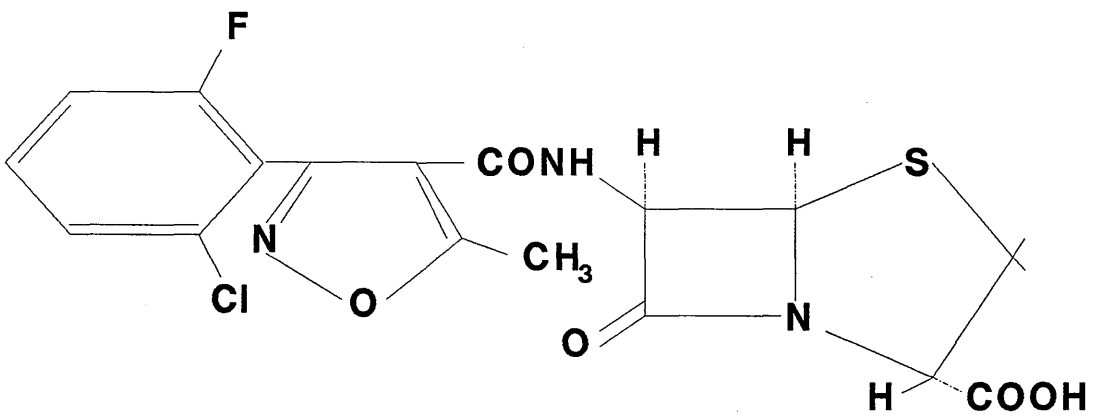
[1]Methicillin-resistance is in fact a misnomer, as the resistance encompasses most, if not all, ß-lactam antibiotics.



Methicillin



Cloxacillin



Flucloxacillin

Figure 1. Structure of methicillin and the isoxazolyl penicillins, cloxacillin and flucloxacillin.

20 % to 3 %, during which time the use of isoxazolyl penicillins and cephalosporins had remained unrestricted (Kayser, 1975).

Since the late 1970s there has been a marked resurgence of MRSA, with many isolates resistant to multiple antibiotics, including the aminoglycosides. They have been responsible for significant outbreaks of nosocomial infection world-wide (Marples and Cooke, 1988), especially in Australia (Pavillard *et al*, 1982; Turnidge *et al*, 1989), North America (Locksley *et al*, 1982) and more recently in many parts of Europe, including Ireland (Cafferkey *et al*, 1985; Morgan and Harte-Barry, 1989) and Great Britain (Cooke *et al*, 1986, Marples, 1988). In addition significant numbers of isolates have been reported from a number of other countries, including Greece, France, Portugal, Italy and the Middle East (Casewell, 1986; Marples, 1988; Maple *et al*, 1989).

These new MRSA have been shown by a variety of techniques, ranging from phage-typing to complex genetic analyses to differ from their predecessors (Townsend *et al*, 1985b; Vickery *et al*, 1986). What is perhaps more interesting is that in contrast with the "old" MRSA where there was evidence of a common clonal origin (Lacey and Grinstead, 1973), a number of studies have now shown significant differences can also occur between strains from different areas (Grubb *et al*, 1985; Marples *et al*, 1986; Townsend *et al*, 1987; Jordens and Hall, 1988; Carroll *et al*, 1989). This suggests that whilst the global spread of a single MRSA clone and its derivatives may occur (Humphreys *et al*, 1990), the present problem

may additionally reflect the local evolution *de novo* of MRSA at multiple sites due to similar selection pressures acting upon endogenous methicillin-sensitive strains.

MRSA IN SCOTLAND

Few MRSA were seen in Scotland before 1984. An initial report by Barrie in Kirkcaldy of 12 isolations of MRSA from clinical infections during 1975 (Barrie, 1976), prompted a request from the Communicable Diseases Scotland Unit (CDS) for information from other laboratories. This study showed that MRSA were mainly present in three centres; Fife, Dumfries Royal Infirmary (where MRSA accounted for 7 % of *Staph. aureus* isolates), and Edinburgh Royal Infirmary. The majority of these isolates were phage type 77 (CDS, 1977). By 1981 MRSA were reckoned to account for 1 % of all *Staph. aureus* isolates in Scotland (White, 1982). A postal survey in 1983 by the Hospital Infection Society revealed that MRSA accounted for between 1 and 4 % of *Staph. aureus* in Scottish hospitals, a figure similar to that found in the rest of the United Kingdom (Cooke *et al*, 1986).

These initial Scottish MRSA have largely been superseded by other MRSA, which demonstrate variation from their earlier counterparts in a number of properties, including antibiogram, biotype and phage type. In the West of Scotland the majority of strains appear similar to, or to be derived from, MRSA first isolated in Glasgow Royal Infirmary (GRI), whereas in other parts of the

country a diversity of strains has been found. In the first report of the Scottish MRSA Study Group, established in July 1986, isolates from GRI accounted for 119 of the 214 MRSA reported (Scottish MRSA Study Group, 1987). The GRI isolates appeared initially as two groups: aminoglycoside-sensitive MRSA derived from the Dermatology wards, and aminoglycoside-resistant MRSA from the Regional Burns Unit. The second Study-group report still showed the majority of MRSA to have been isolated in Glasgow, but with an increasing number of reports from other areas, particularly those with a major tertiary referral centre (Scottish MRSA Study Group, 1988). In the period from the start of 1984 until the end of 1989, there were reports of isolation of MRSA from 1275 patients, with only two of the fifteen health boards remaining MRSA-free. Of these isolations, 732 were from GRI, where MRSA continue to account for around 4 % of all *Staph. aureus* isolations (D R Baird, personal communication).

MECHANISM OF METHICILLIN-RESISTANCE

The ß-lactamase stable penicillins and cephalosporins exert their action in a similar way to penicillin G. Carboxypeptidase and transpeptidase enzymes which are essential to cell wall peptidoglycan synthesis are covalently bound and therefore inhibited (Hammond and Lambert, 1981). Studies of these enzymes, which are collectively referred to as penicillin-binding proteins (PBPs) in *Staph. aureus* have demonstrated four or five distinct species (Table I) (Wyke, 1984; Canepari *et al*, 1985).

Table I. Penicillin binding proteins (PBPs) of *Staphylococcus aureus*

PBP	Molecular weight
PBP1	79,000-87,000
PBP2	73,000-80,000
PBP3	70,000-75,000
PBP3'	70,000
PBP4	41,000-46,000

In contrast to resistance to penicillin G, which is due to degradation of the antibiotic by a bacterial ß-lactamase, resistance to methicillin is intrinsic and is mediated by means of the synthesis of an altered PBP (2' or 2a) of molecular weight 74,000-78,000 which has a greatly reduced affinity for ß-lactam antibiotics (Lyon and Skurray, 1987). Methicillin-resistance is encoded by the *mec* gene which is chromosomally located, as determined by successful transformation of resistance by chromosomal, but not by plasmid, DNA (Sjöström *et al*, 1975). The chromosomal location of this determinant has been genetically mapped (Kuhl *et al*, 1978). The control of expression of this element is complex, involving both an additional distinct chromosomal locus and regulation by a ß-lactamase determinant (possibly mediating an effect via the *bla*I ß-lactamase repressor gene). In addition, the observed decreased PBP 2' expression in acidic conditions and increased expression at 32°C, coupled with the thermolability of the protein, help explain the characteristic heterogeneous nature of methicillin-resistance in clinical isolates of *Staph. aureus* (Lyon and Skurray, 1987).

EPIDEMIOLOGY OF MRSA INFECTION

The study of the origin and spread of a microorganism requires an adequate typing method, and a number have been described to study the epidemiology of MRSA. Before considering some of these methods in more detail, it is perhaps worth considering some of the factors involved in the evaluation and comparison of typing strategies. The key elements of a typing system are typability, reproducibility and discrimination. Typability and reproducibility are reasonably straightforward to assess: typability is simply the percentage of distinct bacterial strains which can be assigned a positive typing marker by the method, and reproducibility is the percentage of strains which yield the same result on repeated testing. Discrimination however, is determined both by the number of discrete types defined and the relative frequencies with which these are detected in the population under study. Although Hunter and Gaston (1988) have suggested the adoption of a discriminatory index derived from these two parameters, based on Simpson's Index of diversity, objective assessments of the discriminatory ability of a typing system have not been widely adopted.

There are other factors which serve to confound objective comparisons of typing systems. Although apparently similar methods may be employed at different centres, there are often significant, and sometimes undisclosed, variations in technique which limit the comparability of the data generated. In addition,

typing methods are often developed to address specific local problems, and as a result may reflect the properties and behaviour of local isolates. Even in the local context, there is an understandable tendency to concentrate on isolates of interest, such as "epidemic" strains, without fully assessing the characteristics of the "background" population of the same organism.

Clearly, the introduction of any method for widespread use requires careful standardisation and validation in suitable representative collections of the organism. To justify the initial outlay any new method must demonstrate a significant advantage, by providing more useful information, faster results, or a saving in resources.

Traditional Methods

Phage typing has been employed for more than 40 years as the routine method to study the epidemiology of infection with *Staph. aureus*. Fisk (1942a, 1942b) was the first to show that *Staph. aureus* carried phages which could be detected by cross-culture of pairs of strains and propagated on the sensitive member of the pair by strain-specific lysis. Wilson and Atkinson (1945) subsequently established a set of phages for the classification of *Staph. aureus*, and in 1953 an International Subcommittee was formed to standardize the methodology. Since that time the method has been extensively developed and, although originally important as a means of distinguishing between strains of staphylococci in small short-

lived local epidemics, has been of immense value in the study of national and indeed international trends in the spread of staphylococcal infections (Parker, 1983). Perhaps the best known example of this latter phenomenon was the identification and documentation of the subsequent spread of the uniquely pathogenic strains belonging to phage type 80/81 (Parker, 1983).

Unfortunately the application of phage typing to the study of MRSA has been rather disappointing. The original MRSA strains of the 60s and 70s had a variety of phage-typing patterns (Parker and Hewitt, 1970) in spite of genetic evidence to suggest a common clonal origin (Lacey, 1972; Lacey and Grinsted, 1973), probably reflecting their variable lysogenic state. However, of more concern is the fact that many of the MRSA strains encountered at present are poorly typed by the current International Set of phages (Marples *et al*, 1986; Marples, 1988; Richardson *et al*, 1988). In addition, it has been demonstrated previously that the typability of epidemic strains of *Staph. aureus* may decrease with time (Jevons and Parker, 1964); such a phenomenon has been described for EMRSA isolates (Marples *et al*, 1986). Although the use of supplementary phages may increase the number of typable strains (Vickery *et al*, 1986; Richardson *et al*, 1988), it has become clear that phage-typing alone is inadequate for the investigation of outbreaks of MRSA infection.

Other traditional approaches to staphylococcal epidemiology have included resistotyping (Elek and Moryson, 1974) in which strains are differentiated according to their degree of

resistance to a variety of chemicals and antibiotics and their biotype. Resistotype tests, particularly for resistance to heavy metals, are often used now to detect plasmid-encoded phenotypic markers (Emslie *et al*, 1985; Townsend *et al*, 1985a). Antibiograms alone do not tend to produce sufficiently detailed discrimination for epidemiological purposes. However, a typing system proposed recently for MRSA uses antibiotic-sensitivity test results in conjunction with sensitivity to various chemicals and this shows some promise for routine application (Gillespie *et al*, 1990). Cookson *et al* (1986) have described a biotyping system for MRSA based upon a modification of the technique of Andrew and Symons (1982). This system tests α- and ß-haemolysin production, proteolysis, pigmentation, Tween 80 hydrolysis, lipase and lecithinase production, and was found to be useful in the initial screening of MRSA isolates. Simple biochemical tests (Peacock *et al*, 1981; Vickery *et al*, 1988) and pigmentation (Lacey and Stokes, 1979) appear to be of limited value only, in strain discrimination.

Recent developments

In recent years a great deal of attention has focussed on the application of modern biochemical and molecular biological techniques to the study of the epidemiology of the agents of infectious disease. The use of such methods in this context has been reviewed recently (Goldmann, 1987; Hawkey, 1987). In essence, all of these methods separate large organism-derived biological macromolecules by gel electrophoresis to yield a pattern which is

characteristic of the strain; they differ in the nature of the analyte, which may be protein, plasmid or nucleic acid fragment, and the means of demonstrating the resulting patterns.

Proteins

Branger and Goullet (1987, 1989) characterised MRSA by means of the polymorphism of esterase enzymes. The enzymes were separated by means of polyacrylamide agarose gel electrophoresis. The esterases were subsequently "stained" in the gel by means of their interaction with a variety of synthetic substrates. Depending upon the pattern of activities demonstrated, and their selective inhibition by various substrate analogues, they were able to assign isolates from different countries and outbreaks to one of a number of distinct zymotypes. Again, their results supported the hypothesis that there had been global spread of some MRSA types, whereas others had evolved locally. Some zymotypes were unique to specific locations, and others belonged to the same type as local methicillin-sensitive strains. Although excellent reproducibility, typability and discrimination were possible, the procedure was both lengthy and technically complex.

Analysis of whole-cell protein profiles of MRSA generated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) has also been used to study the epidemiology of MRSA infection. However, whether performed by semi-automated densitometric examination of ^{35}S-methionine labelled polypeptides

in an automated bacterial identification system (AMBIS), or manually by means of Coomassie-blue stained preparations, discrimination of different strains was poor (Stephenson *et al*, 1986; Thomson-Carter and Pennington, 1989).

Burnie and Matthews (1987) first suggested the use of Western blot analysis of whole-cell polypeptides as a typing method for bacterial pathogens. Polypeptides are separated by means of SDS-PAGE and "blotted" onto nitrocellulose filters (Towbin *et al*, 1979). They are then "probed", usually with hyperimmune rabbit antisera raised against the pathogenic microorganism. After washing, specifically bound antibodies are detected by the application of anti-rabbit globulin conjugated to a fluorescent or enzymatic marker which enables the antigenic polypeptide bands to be demonstrated. Recognition of antigenic variation within a subset of the total polypeptide bands present permits greater discrimination than SDS-PAGE analysis alone. This technique has been applied successfully to a range of microorganisms including *Candida albicans*, coagulase-negative staphylococci and *Clostridium difficile* (Lee *et al*, 1986; Burnie *et al*, 1988; McKay *et al*, 1989). MRSA can be typed successfully by this method (Lee and Burnie, 1988), and good correlation has been shown with the results of a variety of other methods including phage-typing, plasmid profile analysis and restriction enzyme digestion of chromosomal DNA (Gaston *et al*, 1988; Burnie *et al*, 1989).

Nucleic acids

The genetic basis of antibiotic resistance in staphylococci has long been a subject of interest, speculation and intensive research activity by microbiologists (Lacey, 1972, 1975; Shalita *et al*, 1980). It is hardly surprising that the application of modern molecular techniques has resulted in an enormous increase in information about these processes. This work has been reviewed recently in some detail by Lyon and Skurray (1987). The analysis of chromosomal and extrachromosomal DNA has also been an excellent research method for the molecular epidemiologist (Platt, 1983).

Plasmid profiles

Plasmids are circular molecules of supercoiled DNA which exist independently of the main chromosome within the cytoplasm of the bacterial cell. They are capable of autonomous replication and segregate during the process of cell division, to become partitioned within the daughter cells. Plasmids have been identified in virtually all bacterial genera. The number of copies of any given plasmid in a single cell varies, and may be as many as 40 for small plasmids, or as few as one if the plasmid is large. A variety of phenotypic traits may be encoded by plasmids: of greatest clinical importance is antibiotic resistance. In general plasmid-encoded traits are not essential for host survival, although they may confer selective advantages upon the organism. However, the function of the majority of plasmids remains unknown; these are termed cryptic

plasmids (Broda, 1979; Novick, 1980). Different plasmid species may co-exist within the same cell, but related plasmids tend to be incompatible, and such incompatibility provides the basis of a means of classification (Datta, 1977). Staphylococcal plasmids can be exchanged between organisms by bacteriophage transduction, transformation and processes analogous to conjugation (Lyon and Skurray, 1987). In addition inter-species transfer of plasmids has been demonstrated, for example between *Staph. aureus* and coagulase-negative staphylococci (Forbes and Schaberg, 1983). The relative importance of these various phenomena *in vivo* is poorly understood.

Until about 10 years ago, the prolonged and complicated procedures for the detection and preparation of plasmid DNA, which required large culture volumes and density-gradient centrifugation, precluded the widespread application of plasmid analysis as an epidemiological tool in medical microbiology. However, the advent of rapid, simple techniques for the isolation of plasmid DNA provided a method for the analysis of large numbers of clinical isolates. (Birnboim and Doly, 1979; Platt and Sommerville, 1981). The number and size of plasmids harboured by a given bacterial strain, can be demonstrated by agarose gel electrophoresis of the extracted plasmid DNA and staining with the intercalating dye ethidium bromide; this is referred to as a *plasmid profile*. It is the comparison of such plasmid profiles from a series of isolates which provides the basis of a typing scheme for use in epidemiological

studies. It is obvious that if an isolate is plasmid-free, it cannot be typed by this method, and if certain plasmids are present invariably in all isolates of an organism, the value of those plasmids as epidemiological markers is correspondingly diminished. For these reasons when developing a typing scheme it is necessary at the outset to determine the relative abundance and distribution of plasmids within collections of organisms representative of the population to be studied (Platt *et al*, 1984, 1986a). Plasmids interact with their host organisms in a dynamic way and as a result may be lost, gained and undergo molecular rearrangement (Hawkey, 1987). The stability of any given plasmid species depends on two major conflicting factors: the benefits for the survival of the host organism conferred by properties encoded by the plasmid, balanced against the disadvantages imposed by the biosynthetic burden due to its continued carriage.

Plasmid profile analysis has been successfully employed in studies of the epidemiology of infection with a range of Gram-negative and Gram-positive bacteria (Farrar, 1983; John *et al*, 1983; Schaberg and Zervos, 1986). The technique has been of particular value in the study of outbreaks of salmonellosis (Taylor *et al*, 1982; Threlfall *et al*, 1986) and coagulase-negative staphylococcal infection (Archer *et al*, 1984). Various groups have employed the method as part of their investigation of the epidemiology of MRSA infection (Cookson *et al*, 1986; Gelmi *et al*, 1987; Rhinehart *et al*, 1987; Gaston *et al*, 1988), although in all but one case (Rhinehart *et*

al, 1987) plasmid profile data alone was of limited value, due to the frequent finding of isolates with similar plasmid profiles, and plasmids of similar molecular weights. None of these studies attempted to compare the plasmid profiles of MRSA with those of methicillin-sensitive strains present in the same environment.

Restriction enzyme fragmentation patterns

Plasmids of similar size can often be difficult to differentiate by means of plasmid profiles alone. Agarose gel electrophoresis is also relatively inaccurate in determining the size of large plasmids (Hawkey, 1987). To solve these problems it is necessary to develop a method which can differentiate plasmid species, especially when they are of similar molecular size. Diversity depends ultimately on variations in the DNA sequences of the plasmids, and the technique to be employed must be able to demonstrate such differences in base sequences.

Type II restriction endonucleases are enzymes which recognise specific, palindromic sequences in double stranded DNA molecules, probably by virtue of the tertiary structure of the molecules comprising the sequence, and catalyse the hydrolysis of the DNA strands at sites usually a few bases apart (Table II) (Platt, 1983; Hawkey, 1987). When plasmid DNA is subjected to restriction endonuclease digestion a characteristic series of linear DNA fragments, ultimately determined by the DNA sequence of the plasmid, is produced. Separation of these fragments by agarose gel

electrophoresis and staining with ethidium bromide produces a characteristic restriction enzyme fragmentation pattern (REFP) or "fingerprint"[2] (Platt, 1983).

Table II. Examples of palindromic restriction endonuclease enzyme cleavage sites

Producer organism	Enzyme	Base sequence
Escherichia coli	*Eco*R1	5'-G↓AATTC-3' 3'-CTTAA↑G-5'
Bacillus globigii	*Bgl*I	5'-GCCN↓NNNNGGC-3' 3'-CGGNNNN↑NCCG-5'
	*Bgl*II	5'-A↓GATCT-3' 3'-TCTAG↑A-5'
Haemophilus influenzae	*Hind*III	5'-A↓AGCTT-3' 3'-TTCGA↑A-5'
Serratia marcescens	*Sma*I	5'-CCC↓GGG-3' 3'-GGG↑CCC-5'

N = any nucleotide
↑ and ↓ indicate site of hydrolysis

Ideally, the number of fragments generated should be sufficient for specificity, but production of an excess should be avoided so that coincidental matching of bands does not take place. Comparison of REFPs generated by a second different restriction enzyme is usually sufficient to confirm the identity of a pair of

[2]Fingerprint in this context should not be confused with the forensic technique of DNA fingerprinting. This latter technique for detection of restriction fragment length polymorphisms (RFLPs) is analogous, but only a subset of fragments is visualised as a result of hybridisation of probe DNA subsequent to Southern blotting of test DNA fragments to nitrocellulose membranes.

plasmids thought to be identical on the basis of initial matching REFPs. Unfortunately standardised restriction enzyme digestion strategies which would facilitate comparison of plasmid data from different centres have not yet been adopted widely (Platt *et al*, 1986b).

The application of this method is extremely powerful in studying the epidemiology of infections. John *et al* (1983) were able to show that two episodes of *Klebsiella pneumoniae* cross infection on separate wards in the same hospital, due to strains which all harboured a large (170 kb) plasmid, were unrelated because there were differences in the REFPs of the plasmids. The techniques have been applied very widely to a number of bacterial species, including some for which no previous typing schemes existed including *Citrobacter diversus*, *Legionella pneumophila* and *Enterobacter cloacae* (Goldmann, 1987; Hawkey, 1987).

In the study of MRSA, REFP analysis of plasmid DNA has been used to investigate the evolution and spread of a number of resistance plasmids (Dowd *et al*, 1983; Townsend *et al*, 1984, 1985a, 1985b; Kayser *et al*, 1986; Lyon and Skurray, 1987). Furthermore, the molecular epidemiology of various MRSA outbreaks has been analysed by this method; REFP typing compares favourably with other methods (Gelmi *et al*, 1987; Rhinehart *et al*, 1987; Gaston *et al*, 1988).

Chromosomal REFPs

Although plasmid REFP analysis is a powerful tool for epidemiological studies, it suffers from the important limitation that the isolates under investigation must harbour plasmids, and that they must be stable and sufficiently diverse. Furthermore, the analysis of isolates which carry many plasmids can be difficult, and may sometimes require the prior segregation of plasmids. These problems can be avoided by preparing REFPs of chromosomal DNA.

The chromosomal DNA sequence found in different strains of the same organism will vary to some extent as a result of DNA rearrangements: these involve a variety of legitimate and illegitimate recombination events including deletions, duplications, inversions and transpositions. Thus, in a manner analogous to plasmid REFPs, digestion by restriction endonucleases should yield a unique series of linear DNA fragments, which will produce a characteristic pattern for that strain after agarose gel electrophoresis and ethidium bromide staining. These chromosomal REFPs have been employed for typing a variety of bacteria including *Legionella pneumophila* (Van Ketel *et al*, 1984), *Neisseria meningitidis* (Bjorvatn *et al*, 1984), *Campylobacter jejuni* (Bradbury *et al*, 1984) and *Helicobacter pylori* (Langenberg *et al*, 1986). Again, the technique has been used to study the epidemiology of MRSA infection (Jordens and Hall, 1988), and was found to compare favourably with data derived from immunoblotting (Burnie *et al*, 1989).

Compared with plasmid DNA chromosomal DNA is always present and is relatively stable, yet the technique lacks the refinement of plasmid analysis. The main difficulties are the very large number and range of sizes of fragments generated by restriction endonuclease digestion of chromosomal DNA. These fragments are difficult to resolve, coincidental matching of fragments of similar size is more likely to occur, and small differences are easily overlooked during visual inspection. In addition, if plasmid DNA is present, this may have unpredictable effects on the chromosomal REFP. Improved automated scanning techniques utilising laser or ultra-violet densitometry, coupled with microcomputer analysis, should help resolve some of these difficulties (Hawkey, 1987). More refined methods of electrophoresis using field inversion and pulse techniques may improve fragment resolution and facilitate the study of fragments in more discrete size ranges.

DNA probes

DNA probes are single-stranded DNA molecules, which can be labelled by radiometric or non-radiometric means, and which can hybridise with complementary nucleic acid sequences in test samples of DNA. If such a probe is applied to restriction endonuclease digested DNA fragments which have been transferred to nitrocellulose filters by the "blotting" technique of Southern (1975), it will hybridise with those fragments which contain sequences complementary to those of the probe. As a result these

fragments may be visualised by autoradiography or colourimetrically if the non-radiometric biotin-avidin system is used (Hawkey, 1987).

Probes have been employed in two main ways to investigate epidemiological problems. Firstly, probes for specific genes characteristic of the test organism may be synthesised; examples include toxin or antibiotic inactivating enzyme genes. These probes can be used to identify the chromosomal restriction enzyme digest fragment on which the gene is harboured. A study of variations in the fragments which bear the gene in different strains provides a useful epidemiological marker. This technique has been used to study the epidemiology of infections due to *Vibrio cholerae* and enterotoxigenic strains of *Escherichia coli* (Wachsmuth, 1986). In an analogous manner, families of probes may be synthesised for a given organism, representing a variety of chromosomal genes and differences in the patterns obtained after hybridisation compared (Hawkey, 1987). A DNA probe derived from the PBP 2A gene of MRSA has been used to detect methicillin-resistance by means of dot-blot filter hybridisation (Archer and Pennell, 1990).

The second, and more generally applicable technique, is to prepare probes which will hybridise with sequences which are widely conserved across bacterial genera, and which occur in multiple copies on the bacterial chromosome. An example of a set of genes satisfying these criteria in bacteria are the genes encoding ribosomal RNA (rRNA). Following restriction endonuclease

digestion, agarose gel electrophoresis, and Southern blotting as described above, DNA fragments carrying rRNA genes (rDNA) can be demonstrated by using a labelled *Escherichia coli* 16 + 23S rRNA probe. The probe will hybridise with multiple fragments, and variations in the underlying DNA sequence of the strains analysed will be reflected in different fragment patterns (Grimont and Grimont, 1986). In some respects this process is analogous to forensic human DNA fingerprinting, which uses the variation of restriction fragment lengths bearing highly conserved and highly repeated "minisatellite" sequences in a similar manner, to determine the genetic identity/non-identity of individuals (Gill *et al*, 1985).

This is a technique of great potential in epidemiology. Since the probe sequence is widespread amongst all bacterial genera, a single probe/set of probes, can be used to type a huge range of organisms; the specificity of the system derives from the pattern produced, not the detection of a species-specific marker. Regardless of organism, the same techniques and reagents are employed, eliminating the need for a variety of methods for different organisms. The system is currently being developed by Grimont at the Pasteur Institute, and has already been applied to typing a wide range of phylogenetically diverse organisms, including several species for which no satisfactory typing system had been developed (Irino *et al*, 1988; Grimont *et al*, 1989). DNA probes have yet to be employed in the study of the epidemiology of MRSA infection,

although the availability of suitable probes makes such application virtually inevitable.

PATHOGENICITY OF *STAPHYLOCOCCUS AUREUS*

As has been mentioned, *Staph. aureus* possesses a very wide armamentarium of pathogenic mechanisms. These virulence factors can be subdivided according to their location as either cell wall associated or extracellular.

Cell wall associated factors

The cell-wall peptidoglycan has a number of important biological properties: it can stimulate endogenous pyrogen production by human monocytes, has endotoxin-like activity and can activate complement. In addition it can attract polymorphonuclear leucocytes and is able, in association with teichoic acids, to elicit the production of antibodies (Kaplan and Tenenbaum, 1982).

Protein A is a cell-wall protein with molecular weight of 42,000, which is present in over 90 % of *Staph. aureus* isolates. Protein A can activate complement and is capable of inducing immediate and delayed type hypersensitivity. In addition it is chemotactic for leucocytes, can activate lymphocytes and macrophages and binds the F_c receptor of all human IgG subclasses, except IgG3 (Forsgren *et al*, 1983).

Some strains of *Staph. aureus* seem capable of elaborating a polysaccharide capsule. The incidence of capsule production has

been variously reported in between 0.6 % and 18.4 % of strains, depending on the freshness of the clinical isolates examined. It seems likely that these figures may well be higher for organisms growing in vivo. Capsulated strains are more lethal for mice and much less susceptible to phagocytosis. This latter phenomenon is thought to be due to interference with the interaction of C3b bound to the bacterial cell-wall and the C3b receptor on the cell membrane of the phagocytic cell (Wilkinson, 1983).

Extracellular products

Staph. aureus produces and secretes a number of enzymes and toxins which have been variously implicated in the pathogenicity and invasiveness of the organism. The difficulties in elucidating their precise role are compounded by the technical problems in purifying these unstable substances. As a result there is still much controversy about their individuality, specificity and ultimately their *in vivo* activity. It is likely that the advent of modern techniques of molecular biology will permit the preparation of more highly purified material, both by means of cloning and controlled expression of toxin genes, and by improved techniques for molecular separation.

Enzymes

Table III lists many of the enzymes known to be secreted by *Staph. aureus*. The majority of these enzymes degrade macromolecular substrates including proteins, nucleic acids, lipids

and polysaccharides to provide low molecular weight nutrients for bacterial growth. Although there is a high correlation between pathogenicity of *Staph. aureus* and production of extracellular enzymes, there is no evidence to indicate that the ability of staphylococci to cause disease is related to any single substance (Arvidson, 1983).

Table III. Extracellular enzymes of *Staphylococcus aureus*

Coagulase
Nuclease
Lipase
Catalase
Phosphatase
Endopeptidase
Hyaluronate lyase
Lysophosphatidase
Staphylokinase
Proteinases
Phosphatidylcholine: cholesterol *o*-acyl-transferase
Endopeptidase
Endo-β-N-acetylglucosaminidase
N-acetylmuramyl-L-alanine amidase

Those enzymes which may be most closely associated with pathogenicity include coagulase, hyaluronidase, nuclease, lipase and catalase. Coagulase, which causes plasma to clot by activation of the final steps of the coagulation cascade, is thought to interfere with opsonophagocytosis. Hyaluronidase may facilitate local tissue invasion by digestion of acid mucopolysaccharides in the acellular connective tissue matrix. Staphylococcal nuclease, which possesses both exo- and endonuclease activity, is involved in the degradation of nucleic acids. Lipase activity may play some role in the formation of local suppurative lesions, and the dissemination of

infections (Noble and White, 1983; Arvidson, 1983). Catalase, which catalyses the breakdown of hydrogen peroxide into water and oxygen, is thought to play a role in the neutralisation of toxic oxygen radicals synthesised by phagocytic cells (Mandell, 1975).

Toxins

Staph. aureus produces a variety of extracellular products which are defined as toxins by virtue of their ability to affect host cell function and/or morphology; although some of them mediate their toxic effects by virtue of their enzymatic activity, and might therefore be considered as enzymes. The diseases known to be mediated by specific staphylococcal toxins, which include staphylococcal enterocolitis, staphylococcal food poisoning, exfoliative skin disorders and the toxic shock syndrome, have been reviewed by Bass (Bass, 1982). The major *Staph. aureus* toxins are listed in Table IV.

Table IV. Toxins of *Staphylococcus aureus*.

Alpha haemolysin
Beta haemolysin
Gamma Haemolysin
Delta Haemolysin
Epidermolytic toxins (ETA and ETB)
Enterotoxins (A-E)
Panton-Valentine leucocidin
Toxic shock syndrome toxin (TSST-1)

The epidermolytic toxins are involved in the pathogenesis of the staphylococcal scalded skin syndrome and related conditions (Melish and Glasgow, 1970). There are 2 types, A and B, of

molecular weights 26,500 and 26,000 respectively. Epidermolytic toxin A (ETA) is heat stable and chromosomally encoded, whilst epidermolytic toxin B (ETB) is heat labile and plasmid encoded. Although initially associated with phage group II isolates, subsequent work has demonstrated production of ETA and ETB by a wide range of phage types (Arbuthnott, 1983). The precise molecular mode of action of the toxin remains undefined, although phospholipase activity has been demonstrated in electrofocused toxin (Wiley and Rogolsky, 1985).

Five membrane damaging toxins have been described in *Staph. aureus*. These are α–toxin, β-toxin, γ–toxin, δ–toxin and leucocidin. α–, β–, γ– and δ– toxins are frequently referred to as haemolysins, as their haemolytic activity is the most common method used for their detection *in vitro*. α–toxin is a protein of molecular weight 28,000-39,000. It is a surface active agent, which is capable of lysing a variety of cells by interaction with cell-surface receptors (Mölby, 1983; Thelestam, 1983; Fackrell *et al*, 1985). β–toxin is a Mg^{2+}-dependent sphingomyelinase C of molecular weight 26,000-38,000, which also possesses activity against a wide range of cell types. The observed variation of susceptibility to lysis by β–toxin of erythrocytes from different species reflects the variable proportions of sphingomyelin in their cell membranes (Mölby, 1983; Thelestam, 1983). The mode of action of γ–toxin is poorly defined, although it may be a phospholipase-like activity. It is a protein of molecular weight 29,000-46,000, and demonstrates wide activity

against a range of erythrocytes. γ–toxin is inactivated by acid polysaccharides, including agar, and this fact must be borne in mind when attempting to demonstrate toxin activity in vitro (Mölby, 1983; Thelestam, 1983). δ–toxin is a multimeric protein of molecular weight 200,000. It is a surface active agent which is capable of damaging a large variety of cell types by means of poorly defined mechanisms, which may involve the formation of trans-membrane pores (Freer and Birkbeck, 1982; Mölby, 1983; Thelestam, 1983). Leucocidin is exclusively active against leucocytes, and consists of 2 protein components F and S of molecular weights 32,000 and 31,000 respectively. Membrane damage is inflicted by the activation of an endogenous membrane associated phospholipase A_2 activity (Mölby, 1983; Thelestam, 1983). The biological effects of these toxins has been reviewed in detail by Wadström (1983).

The enterotoxins A, B C_{1-3}, D and E are a series of low molecular weight proteins (25,000-30,000) of similar activity and composition, but which may be identified as separate proteins on the basis of antigenic differences. In addition to their well recognised role in the pathogenesis of staphylococcal food poisoning, enterotoxins have been implicated in other manifestations of staphylococcal disease, including some cases of toxic shock syndrome (Crass and Bergdoll, 1986). Although their precise mode of action is ill defined, both local and central mechanisms have been proposed. They are mitogenic, and following intragastric

administration are associated with an influx of lymphocytes into the gastrointestinal tract (Bergdoll, 1983; Bergdoll, 1985).

Originally designated as enterotoxin F, Toxic Shock Syndrome Toxin 1 (TSST-1), is now recognised as the toxin implicated predominantly in the pathogenesis of toxic shock syndrome. TSST-1 is a protein of molecular weight 24,000, and shows many similarities to the staphylococcal enterotoxins, including the presence of a so-called "cystine-loop" structure (Bergdoll *et al*, 1985). TSST-1 has been shown to stimulate production of interleukin 1 and tumour necrosis factor. Production of TSST-1 by toxigenic strains is greatly suppressed by excess Mg^{2+} ions, and the ability of certain vaginal tampon fibres to act as ion exchangers for Mg^{2+} ions may explain the association of toxic shock syndrome with the use of tampons (Kass and Parsonnet, 1987).

PATHOGENICITY OF MRSA

The pathogenic potential of MRSA with respect to their methicillin-sensitive counterparts has been the subject of much controversy and speculation amongst all those concerned with the control of hospital infection. Lacey and Chopra (1975) were able to demonstrate that the acquisition of antibiotic resistance determinants in *Staph. aureus* was associated with a decrease in virulence for chick embryos. However, Cutler (1979) showed that guinea-pig virulence decreased progressively with the loss of resistance

determinants. In the clinical setting there can be little doubt that in a number of places, including parts of Eastern Australia and the USA, and certain Dublin hospitals, MRSA have indeed been responsible for serious epidemic outbreaks of nosocomial infection (Pavillard *et al*, 1982; Locksley *et al*, 1982; Cafferkey *et al*, 1983; Bacon *et al*, 1987; Preheim *et al*, 1987; Cafferkey *et al*, 1988; Marples, 1988). In the South-East of England, particularly in the Thames NHS regions, the spread of so-called epidemic MRSA (EMRSA), and the elaborate infection control measures undertaken in attempts to curb its spread, have been documented extensively (Cookson *et al*, 1986; Dacre *et al*, 1986; Marples *et al*, 1986; Duckworth *et al*, 1988; Marples and Cooke, 1988). Conversely, others have suggested that the MRSA problem has been exaggerated, and have been correspondingly critical of some of the more elaborate infection control procedures which have been undertaken in attempts to eradicate these organisms (Lacey *et al*, 1986; Lacey, 1987).

Some attempts have been made to examine the pathogenicity of MRSA in an objective fashion. The production of various pathogenicity determinants has been investigated including protein A and coagulase (Lacey *et al*, 1986; Roberts and Gaston, 1987; Jordens *et al*, 1989), lipase (Cookson *et al*, 1986), haemolysin (Humphreys *et al*, 1989; Jordens *et al*, 1989) and enterotoxin (Duckworth and Oppenheim, 1986; Humphreys *et al*, 1989; Jordens *et al*, 1989). Compared to methicillin-sensitive strains some differences have been described such as the low level of protein A

production in EMRSA reported by Roberts and Gaston (1987). However, it would seem reasonable to say that on the available evidence, MRSA are probably no more or no less pathogenic than their methicillin-sensitive counterparts. Indeed French *et al* (1990) have shown in a comparison of Hong Kong strains that MRSA have similar lethality to MSSA, in a mouse model and *in vivo*, and are isolated in similar proportions from deep and superficial clinical sites.

These results accord with clinical experience in GRI, where excess morbidity and mortality due to MRSA have not been seen. The number and severity of infections caused by MRSA correspond to the proportion of *Staph. aureus* isolates in the hospital which these strains represent (D R Baird, personal communication). Global variations in clinical experience of these organisms probably reflect a variety of factors, including differences in patient populations studied, the heterogeneous nature of geographically distinct isolates of MRSA themselves, and variations in antibiotic usage and other selection pressures (French *et al*, 1990).

Regardless of their pathogenicity when compared to other *Staph. aureus*, it is clear that MRSA are responsible for significant infections and that the antibiotics available for treatment are significantly curtailed (Musher and Verner, 1985; Waldvögel, 1986). Vancomycin remains the mainstay of therapy and, in addition to the inevitable anxiety that results from dependence on a single agent, it is both inconvenient to administer (Waldvögel, 1986) and potentially

nephro- and ototoxic (Kucers and Bennett, 1989). These factors together with the undoubted potential of all staphylococci to colonise and to spread in the hospital environment, are sufficient reason to alert hospitals to the continued need for surveillance and control of the spread of MRSA (French *et al*, 1990).

The goal of total eradication is probably unrealistic. They are uniquely adapted to the hospital environment consequent upon the selective pressures of widespread use, and abuse, of broad-spectrum antibiotics. Against such a background, attempts at complete control can be extremely costly in terms of time, manpower and resources (Pearman *et al*, 1985). It may be that infection control strategies will have to rely increasingly on monitoring the spread of MRSA, and intervening only when significant levels of nosocomial infection become apparent, or when particularly vulnerable patient groups are threatened (Lacey, 1987).

CHAPTER 2

Materials and Methods

BACTERIAL STRAINS

Methicillin-sensitive *Staphylococcus aureus* (MSSA)

A total of 163 isolates of MSSA was studied comprising : from GRI patients 50 consecutive blood culture isolates collected during the period January 1985 to May 1986 (BC) and 63 isolates from other routine diagnostic specimens collected between October 1985 and April 1986 (RD). In addition 50 strains cultured from the noses of patients attending a general practitioner's surgery (Balfron Health Centre, Balfron) were collected between April 1986 and July 1986 (GP). Multiple isolates from the same patient were excluded.

Methicillin-resistant *Staphylococcus aureus* (MRSA)

A total of 101 isolates of MRSA from GRI patients were studied. These isolates comprised an initial collection of 53 isolates collected between February 1985 and April 1986 (MRSA(1)), and a second collection of 48 isolates from the period May 1986 to July 1987 (MRSA(2)). Approximately half of the isolates were obtained from the Regional Burns Unit based at GRI; the remainder were isolated from a wide variety of units in the hospital. Multiple isolates from the same patient were again excluded. In addition 3 isolates of epidemic MRSA (EMRSA) from London, two from St. Bartholomew's hospital and one from St. Thomas's hospital were also examined.

ISOLATION AND IDENTIFICATION OF ORGANISMS

Organisms from episodes of bacteraemia and septicaemia were isolated using the radiometric Bactec system (Becton-Dickinson, Cowley) in which the detection of $^{14}CO_2$ released from a ^{14}C-labelled carbon source in the liquid culture medium indicates bacterial growth. Subsequent to their isolation, the organisms were stored in Robertsons cooked meat broth.

Non-blood culture specimens were collected on standard charcoal swabs (Medical Wire and Equipment Company, Corsham) and cultured on Columbia Blood Agar (5 % defibrinated horse blood in Columbia Agar) and incubated at 37°C for 18 hours in air supplemented with 5 % CO_2. Organisms were identified as *Staphylococcus aureus* by their colony morphology and positive results in the coagulase test and Staphaurex latex agglutination test (Wellcome, Dartford) (Duguid, 1989).

ANTIBIOTIC SUSCEPTIBILITY TESTING

Sensitivity to methicillin (10 μg), rifampicin (2 μg), streptomycin (10 μg), kanamycin (30 μg), gentamicin (10 μg), vancomycin (30 μg), trimethoprim (2.5 μg), mupirocin (5 μg), penicillin G (2 μg), erythromycin (5 μg), clindamycin (2 μg), fucidin (10 μg), tetracycline (10 μg), chloramphenicol (10 μg) and teicoplanin (30 μg) was determined by disc-diffusion tests. Five colonies from an overnight culture of the test organism were suspended in a 2 ml volume of Isosensitest broth (Oxoid,

Basingstoke); a 1/300th ml loopful of this suspension was spread out onto an Isosensitest agar plate and antibiotic discs applied. Sensitivity-test plates were incubated for 18 hours at 37°C in air (30°C in the case of methicillin). The radii of the resultant inhibition zones were compared with critical radii (Rc) previously determined for a fully sensitive strain of Staph. aureus (GRI 22686). The critical radii were calculated from Rc = $(x-3\sigma)$, where x was the mean radius obtained from twelve determinations and σ was the standard deviation. If an antibiotic produced an inhibition zone which was less than Rc, the isolate was deemed to be resistant to that agent (Platt *et al*, 1986a).

PHAGE TYPING

Phage typing of 45 MRSA(1) isolates was performed as previously described (Coia *et al*, 1988) by the staphylococcal reference laboratory of the Central Public Health Laboratory (CPHL) Colindale, London. The isolates were typed with the standard international set of phages, and with the additional set used to type MRSA (616, 617, 618, 620, 622, 623, 625, 626, 629 and 630). In addition 34 of the isolates were typed with phages 88A, 90, 83C and 932.

BIOTYPING

Biotyping of 45 MRSA(1) isolates was performed as described previously (Coia *et al*, 1990) by Dr Dugald Baird. Simple

biotyping was based on three properties:- hydrolysis of tween 80, pigmentation on tween 80 agar and urease production.

Tween 80 (1 % v/v) was incorporated in nutrient agar (Oxoid, Basingstoke), and the test organism spread over an area approximately 1 cm diameter. Plates were incubated at 37°C for 2-3 days. A positive test was denoted by the appearance of a halo of fatty acids around the inoculum. This medium enhanced pigmentation of *Staph. aureus*, and isolates were described as gold, buff or cream.

Urease production was tested in 1 ml volumes of brain-heart infusion broth (Oxoid, Basingstoke) containing 2 % urea and 0.0012 % phenol red as indicator. A heavy inoculum was used. After 18-48 hours incubation at 37°C, a positive test was indicated by a deep red colour of the medium. Urease non-producers turned the medium yellow.

PREPARATION OF IMMUNOBLOTS OF EXPORTED PROTEINS

Immunoblotting of exported proteins from 45 MRSA(1) isolates was performed by Dr. F. Thomson-Carter in the University Department of Bacteriology, Aberdeen, as previously described (Coia *et al*, 1990). Isolates were inoculated on to nutrient agar plates and incubated for 24 hours at 37°C. Sufficient growth was harvested from these plates to provide approximately 2 μg bacterial dry weight/ml; this was inoculated into 150 ml of nutrient broth and

incubated for 18 hours at 37°C in an orbital incubator. After centrifugation at 10000 x g for 15 minutes at 4°C, the upper 10 ml of supernatant containing the exported proteins was retained for immunoblot analysis.

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the proteins by a modification of the technique of Laemmli (1970). Samples were prepared by mixing two parts of the supernatant with one part 0.5 M Tris-HCl, pH 6.8 containing SDS 6 % w/v, glycerol 30 % w/v, 2-mercaptoethanol 15 % w/v, and bromophenol blue 0.001 % and boiling for 5 minutes. Samples containing 40 μg protein were loaded into wells in a 3.6 % acrylamide stacking gel over a 10 % acrylamide separating gel. The proteins were separated by electrophoresis in a discontinuous buffer system at 30 mA constant current for 4-5 hours.

Proteins from the gels were blotted on to nitrocellulose membranes in 25 mM Tris-HCl, 192 mM glycine buffer, pH 8.3, containing methanol 20 % v/v (Burnette, 1981). A current of 100 mA applied overnight in a Bio-Rad transblot cell was used to transfer the proteins. A 30 minute incubation in blocking buffer containing newborn calf serum (Gibco-BRL, Paisley) 10 % v/v in phosphate-buffered saline, pH 7.4, Triton X-100 0.2 % v/v (PBS/Triton), served to saturate free protein sites. The nitrocellulose membrane was then incubated in test human plasma diluted 1 in 50 with blocking buffer for 90 minutes. The test human plasma was prepared from a pool known to contain antibodies against a range of

staphylococcal polypeptides (Krikler *et al*, 1986). The membranes were then given three 15 minute washes in PBS/Triton, prior to incubation for 45 minutes with horseradish peroxidase-linked sheep anti-human IgG serum (Scottish Antibody Production Unit, Carluke) diluted 1 in 1000 in blocking buffer. Three more washes in PBS/Triton were then performed and the membranes rinsed in PBS alone prior to staining with diamino-benzidine tetrahydrochloride (BDH, Poole) 0.05 % w/v in 0.1 M Tris-HCl, pH 7.6, containing hydrogen peroxide 0.01 % v/v.

DETERMINATION OF PLASMID PROFILES

Plasmid profiles were determined by a modification of the method of Platt and Sommerville (1981) as described by Coia *et al* (1988). About 25 % of the growth from an overnight culture on nutrient agar at 37°C was harvested using a dry cotton wool swab and suspended in 500 μl of a solution containing 50 mM Tris, 50 mM NaCl and 5 mM disodium EDTA, pH 8.0 (TES) in Eppendorf tubes. The cells were pelleted by centrifugation at 15000 x g for 30 seconds and the supernatant discarded. The cells were resuspended in 400 μl of TES containing 50 mM sucrose (TESS) to which 50 μl of a solution of lysostaphin (1 mg/ml in TES, Sigma Chemicals, Poole) and 50 μl of a solution of lysozyme (40 mg/ml in TES, Sigma Chemicals, Poole) were added. After incubation for 10 minutes at 37°C, 400 μl of a solution of sodium dodecyl sulphate, 10 % w/v (SDS) was added to complete lysis. The lysates were centrifuged for 15 minutes at 15000 x g and the bulky

pellets discarded. One hundred microlitre aliquots of the resulting plasmid-bearing supernatants were loaded onto a vertical agarose gel (0.7 % w/v in 89 mM Tris, 89 mM borate and 1.25 mM EDTA, pH 8.2 (Tris-borate)) and 5 μl of tracker dye (0.05 % w/v bromophenol blue, 0.1 % w/v SDS, 8 mM sodium acetate in 25 % w/v sucrose) added to each well. The plasmids were separated by electrophoresis for 5 hours at 20 V/cm as described by Platt and Sommerville (1981) and Platt *et al* (1984). The gels were stained for 30 minutes in ethidium bromide solution (1.6 μM in Tris-borate), the plasmid bands visualised by ultra-violet light (360 nm) on a transilluminator and photographed using a Polaroid MP4 camera (665 film: 40 second exposure, f 5.6). The molecular sizes of the plasmids were estimated by comparison with plasmids of known size (96, 42, 24 and 4.6 Md isolated from the multiplasmid *Escherichia coli* strain 39R861). The sizes of small plasmids were estimated by comparing their mobility with that of a set of low molecular weight standard supercoiled plasmids (Supercoiled Ladder, Gibco-BRL, Paisley), as described previously by Platt and Taggart (1987).

PREPARATION OF PURIFIED PLASMID DNA

Purified staphylococcal plasmid DNA was prepared by a modification of the method of Platt *et al* (1986b), as described by Coia *et al* (1988). Cultures incubated overnight in 10 ml of brain heart infusion broth (BHI) were centrifuged at 2000 x g for 5 minutes. The cell pellet was resuspended in 1 ml TES buffer, and

each sample then divided into two Eppendorf tubes. Following centrifugation at 15000 x g for 30 seconds, the cell pellets were resuspended in 200 μl of TESS to which 50 μl of lysozyme (40 mg/ml) and 50 μl of lysostaphin (1 mg/ml) were added. Four hundred microlitres of a freshly prepared alkaline SDS solution (1 % SDS, 0.2 M NaOH) was added to each tube, followed by gentle inversion to complete cell lysis. After incubation for 5 minutes at 4°C, 300 μl of 3 M sodium acetate solution was added and the tubes gently inverted until a white precipitate was visible. After thorough vortex mixing the samples were again incubated for 5 minutes at 4°C, prior to centrifugation at 15000 x g for 2 minutes. 0.5 ml of (1:1) phenol/chloroform mixture was added to the resulting supernatants prior to vortex mixing and further centrifugation at 15000 x g for 2 minutes. The upper aqueous layer was retained after centrifugation and 0.5 ml of propan-2-ol added. The samples were allowed to stand for at least 5 minutes at room temperature.

After further centrifugation for 2 minutes at 15000 x g the pellet was resuspended in 100 μl of a solution containing 10 mM Tris and 1 mM EDTA, pH 8.0 (TE). After 5 minutes the duplicate tubes were pooled and 100 μl of 7.5 M ammonium acetate solution added. 0.6 ml of ice-cold 100 % ethanol was then added and the samples kept at -20°C overnight.

After centrifugation for 2 minutes at 15000 x g the resulting pellets were resuspended in 160 μl TE buffer and 18 μl of ribonuclease (1 μg/ml) added. After incubation for 30 minutes at

37°C, 20 μl of 2.5 M sodium chloride solution was added. Once again 0.5 ml of phenol/chloroform mixture was added and the upper aqueous layer separated by centrifugation and retained as before.

0.5 ml of propan-2-ol was added and the tubes allowed to stand a further 5 minutes at room temperature. After centrifugation for 2 minutes at 15000 x g the pellets were resuspended in 100 μl TE buffer and 100 μl 7.5 M ammonium acetate added. After the addition of 0.6 ml of ice-cold 100 % ethanol the tubes were again kept at -20°C overnight. Finally the samples were centrifuged for 2 minutes at 15000 x g and the pellets of purified plasmid DNA resuspended in 60 μl of TE buffer.

RESTRICTION ENDONUCLEASE DIGESTION

The purified plasmid DNA was digested with restriction endonuclease enzymes according to the strategy previously described by Platt *et al* (1986b); the enzymes used were *Pst*I, *Hae*III and *Alu*I (Gibco-BRL, Paisley). The resultant fragments were separated by electrophoresis to generate restriction enzyme fragmentation patterns (REFP). In addition, the enzymes *Ava*II, *Hin*dIII, *Sma*I, *Eco*RI, *Bsp*1286 and *Alu*I (Gibco-BRL, Paisley) were employed in attempts to cleave low molecular weight plasmids present in some of the isolates.

To each reaction tube was added 5 μl of the appropriate reaction buffer for each enzyme, as supplied by the manufacturer (Gibco-BRL, Paisley). Two microlitres of the enzyme (20 units)

and 15-20 μl of purified plasmid DNA solution was then added and the volume made up to a total of 50 μl with sterile distilled water. The reaction mixtures were then incubated at 37°C (30°C for *Sma*I) for 4 hours to facilitate digestion. Molecular weight standards were generated by the substitution of bacteriophage lambda DNA (Gibco-BRL, Paisley) in place of sample DNA in the reaction mixture, and digestion with the enzyme *Pst*I. After incubation 5 μl of tracker dye was added to each tube and the mixtures loaded into the wells of a horizontal agarose gel (0.8 % in Tris-borate buffer containing 0.3 μg/ml ethidium bromide). The restriction fragments were separated by electrophoresis for 18 hours at 2 V/cm (18 mA constant current), visualised on the ultra-violet transilluminator, and then photographed as previously described for plasmid profile gels. The sizes of the DNA fragments generated by restriction endonuclease digestion were estimated from a standard curve produced by fitting a robust modified hyperbola to the observed mobilities of the fragments of known molecular weight of bacteriophage lambda DNA digested by the enzyme *Pst*I (Plikaytis *et al*, 1986).

ENTEROTOXIN PRODUCTION

Production of *Staphylococcus aureus* enterotoxins A, B, C, and D by 152 MSSA and 49 MRSA(1) isolates was determined by reverse passive latex agglutination (RPLA), using commercial SET-RPLA kit tests (Oxoid, Basingstoke), according to the manufacturers instructions (Humphreys *et al*, 1989). Culture supernatants of test organisms were prepared as described previously for

immunoblotting. Twenty-five microlitres of phosphate buffered saline containing 0.5 % w/v bovine serum albumin was dispensed into each well of 5 rows of a V-bottomed microtitre plate. Twenty-five microlitres of the test culture supernatant was then dispensed into the first well of each of the five rows and doubly diluted. The dilutions were not extended into the last well of each row. To each well of the first row was added 25 μl of latex sensitised with rabbit anti-staphylococcal enterotoxin A IgG antibodies. In an analogous fashion 25 μl of anti-enterotoxin B sensitised latex was added to each well of row 2, 25 μl of anti-enterotoxin C sensitised latex to each well of row 3, 25 μl of anti-enterotoxin D sensitised latex to each well of row 4, and 25 μl of a control latex suspension coated with non-immune rabbit globulins to each well of row 5. The contents were mixed thoroughly by micromixer, covered, and left undisturbed on a vibration-free surface at room temperature for 20-24 hours. Each of the test wells (rows 1-4) was then examined for agglutination which indicated the presence of the respective enterotoxin (A, B, C or D). In this manner the number and type of enterotoxins produced by each isolate could be detected. Non-agglutination in row 5 and in the last well of rows 1-5 served to exclude spurious non-specific agglutination and auto-agglutination, respectively.

DETECTION OF HAEMOLYSINS

Staphylococcus aureus α–, β–, γ– and δ–haemolysin production by 152 MSSA and 49 MRSA(1) isolates was detected by

a modification of methods described elsewhere, (Chao *et al*, 1978; Humphreys *et al*, 1988). Rabbit anti-α antiserum and δ-haemolysin were supplied by Dr. T. H. Birkbeck, Department of Microbiology, University of Glasgow.

Five colonies of each organism under investigation were inoculated into 1 ml volumes of BHI broth and incubated for 18 hours at 37°C in air. The organisms to be tested for α–, β–, γ– and δ–haemolysins were then inoculated in batches of 20, using a multi-point apparatus, onto 3 nutrient agar plates. Organisms to be tested for γ–haemolysin were inoculated in an analogous fashion onto a nutrient agarose plate. The plates were then incubated for 18 hours at 37°C in air.

α–haemolysin

Production of α–haemolysin was detected as follows. The nutrient agar plates were overlaid with 5 ml of 5 % v/v rabbit blood agar. After the agar had set, 2 mm diameter wells were cut in the agar in the spaces between the test strains. Into these wells were dispensed 5 μl aliquots of rabbit anti-α–haemolysin antiserum. After incubation for 24 hours in air at 37°C, the plates were examined for haemolysis, with specific inhibition of α–haemolysin activity around the wells containing antiserum.

β–haemolysin

The following procedure was used to detect β–haemolysin activity. The nutrient agar plates were overlaid with 5 ml of 5 % v/v sheep blood agar. After the agar had set, 2 mm diameter wells were cut in the agar in the spaces between the test strains. Into these wells were dispensed 5 μl aliquots of δ–haemolysin. After incubation for 24 hours in air at 37°C, the plates were examined for haemolysis due to β–haemolysin, which could be recognised by enhancement around the wells containing δ–haemolysin.

γ–haemolysin

Production of γ–haemolysin was determined in an analogous fashion to that used for α–haemolysin. The nutrient agarose plates were overlaid with 5 ml of 5 % v/v rabbit blood agarose. After the agar had set, 2 mm diameter wells were cut in the agar in the spaces between the test strains. Into these wells were dispensed 5 μl aliquots of rabbit anti-α–haemolysin antiserum. After incubation for 24 hours in air at 37°C, the plates were examined for haemolysis. Spurious α–haemolysin activity was inhibited by the specific antiserum.

δ–haemolysin

Production of δ–haemolysin was determined as follows. The nutrient agar plates were overlaid with 5 ml of 5 % v/v cod blood agar. The plates were then incubated for 4-8 hours at 4°C and observed for haemolysis.

STATISTICAL ANALYSIS

The overall similarity of any two isolates represented by either immunoblot profile or REFP was determined by calculation of the similarity co-efficient of Dice (S_D) (Dice, 1945), as follows:-

$$\text{Average similarity} = \frac{\text{Number of matching bands x 2}}{\text{Total number of bands in both isolates.}}$$

The distribution of immunoblot types within each REFP group, the distribution of biotypes within each of the major REFP/Immunoblot groups and the distribution of enterotoxins and haemolysins amongst the various collections of organisms was compared by means of the Chi-squared test (Siegel, 1956).

CHAPTER 3

Results

ANTIBIOTIC SUSCEPTIBILITY TESTING

Antibiograms

The complete antibiograms of all the isolates are listed in Appendix 1, together with details of the collection to which they belong. Table V shows the numbers and percentage of isolates in each of the collections resistant to each of the antibiotics tested. Penicillin resistance, as would be expected, was by far the most common resistance seen in the MSSA isolates.

Table V. Numbers of isolates in each collection resistant to individual antibiotics

Antibiotic	Number(%) of isolates resistant to given antibiotic				
	BC (n=50)	GP (n=50)	RD (n=63)	MRSA(1) (n=53)	MRSA(2) (n=48)
Rifampicin	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Streptomycin	1 (2)	2 (4)	2 (3)	25 (47)	28 (58)
Kanamycin	1 (2)	1 (2)	3 (5)	25 (47)	29 (60)
Gentamicin	0 (0)	1 (2)	2 (3)	25 (47)	29 (60)
Vancomycin	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Trimethoprim	0 (0)	1 (2)	1 (2)	2 (4)	0 (0)
Mupirocin	0 (0)	0 (0)	0 (0)	1 (2)	1 (2)
Penicillin	46 (92)	37 (74)	51 (81)	53 (100)	48 (100)
Methicillin	0 (0)	0 (0)	0 (0)	53 (100)	48 (100)
Erythromycin	3 (6)	3 (6)	8 (13)	52 (98)	48 (100)
Clindamycin	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Fucidin	4 (8)	0 (0)	7 (11)	2 (4)	0 (0)
Tetracycline	4 (8)	9 (18)	17 (27)	51 (96)	47 (98)
Chloramphenicol	0 (0)	0 (0)	0 (0)	1 (2)	1 (2)
Teicoplanin	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Table VI shows the percentage of isolates in each group resistant to none, one, two or three or more of the antibiotics tested. All the MRSA were resistant to three or more antibiotics (Figure 2).

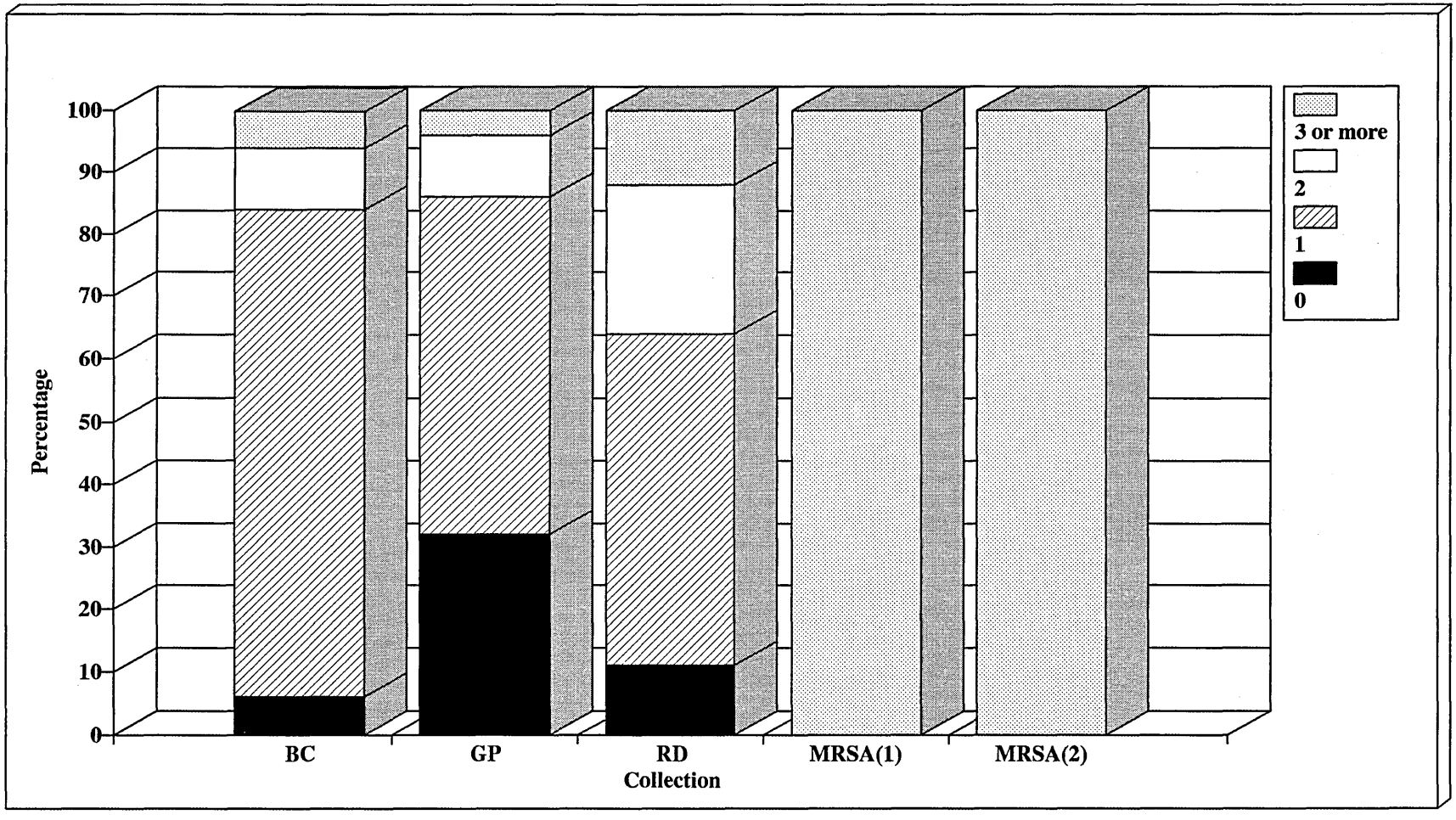


Figure 2. Comparison of the distribution of resistance determinants/isolate of *Staph. aureus* from different sources.

Table VI. Distribution of resistance determinants in isolates from different sources

Resistance determinants	Percentage of isolates resistant to the given number of antibiotics				
	BC	GP	RD	MRSA(1)	MRSA(2)
0	6	32	11	0	0
1	78	54	53	0	0
2	10	10	24	0	0
≥ 3	6	4	12	100	100

Aminoglycoside-resistance

Almost equal numbers of aminoglycoside-sensitive and aminoglycoside-resistant organisms (on the basis of sensitivity or resistance to gentamicin, streptomycin and kanamycin) were found in each of the two MRSA collections. Aminoglycoside-resistant MRSA (MRSA(R)) were predominantly, though by no means exclusively, isolated from patients in the Regional Burns Unit, whereas aminoglycoside-sensitive MRSA (MRSA(S)) were found mainly in the dermatology unit. Two antibiograms accounted for > 90 % of the total combined MRSA isolates (Table VII).

Mupirocin-resistance

One of the MRSA was found to be resistant to the topical antibiotic mupirocin (Baird and Coia, 1987). The isolation of this organism predated the widespread introduction of mupirocin into clinical use.

Table VII. Distribution of MRSA antibiograms

Resistance Markers	Number (%) of isolates with the given antibiogram	
	MRSA(1) (n = 53)	MRSA(2) (n = 48)
P Met Tet Ery	24 (45)	18 (38)
P Met Tet Ery Sm Km Cn	25 (47)	28 (58)
Other	4 (8)	2 (4)

P = Penicillin, Met = Methicillin, Tet = Tetracycline, Ery = Erythromycin, Sm = Streptomycin, Km = Kanamycin, Cn = Gentamicin.

BIOTYPING

Biotyping divided the isolates into 4 groups (A-D) as shown in Table VIII. There was a positive correlation between urease production and gentamicin resistance in our strains. Tween hydrolysis was of particular value in separating gentamicin-resistant strains into two groups arbitrarily designated B and D, as they had similar antibiograms. Although the B strains were more highly resistant to gentamicin they were difficult to distinguish on routine sensitivity testing. Their epidemiology however was quite different; the B strains were confined essentially to the burns unit, whereas the D strains, though common in that unit, were encountered frequently elsewhere.

The strains designated A were first isolated from the dermatology unit, and were at the time of the study, also present in

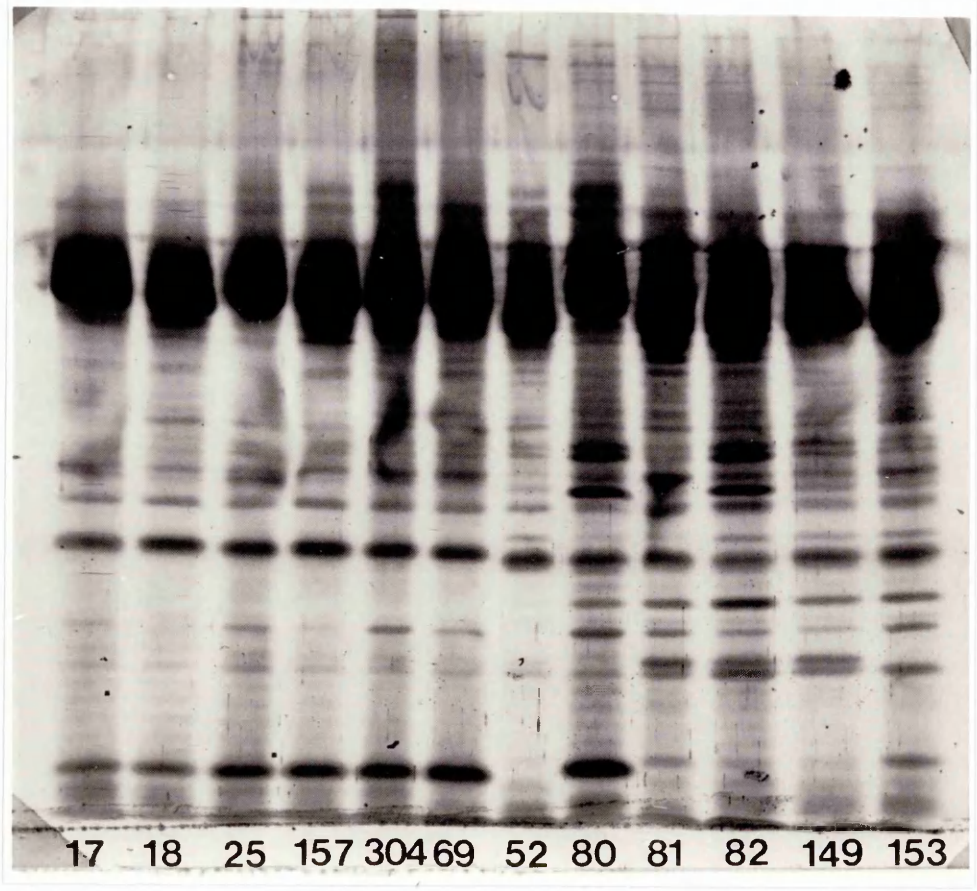
an orthopaedic ward and two associated hospitals. Organisms biotyped as C represented a small, less well defined group, mostly appearing sporadically, and with little tendency to spread.

Table VIII. Biotypes of MRSA

	Biotype			
	A	B	C	D
Tween 80	Negative	Negative	Positive	Positive
Urea	Negative	Positive	Negative	Positive
Pigmentation	Cream	Buff	Variable	Gold
Aminoglycoside	Sensitive	Resistant	Sensitive	Resistant

IMMUNOBLOTTING

Reproducible banding patterns were obtained by the preparation of immunoblots of culture supernatants. Typical results are shown in Figure 3. Each track contained between 33 and 43 bands, with an average of 39. The banding patterns could be divided into two major groups (BLOT1 and BLOT2). Dice analysis of the BLOT1 group of 19 isolates showed an average similarity of 94% (range 85 to 98%). The BLOT2 group comprised 24 isolates with an average similarity of 98% (range 94 to 100%). Comparisons between these groups demonstrated a lower inter-group average similarity of 61% (range 57 to 83%). A third minor group (BLOT3) comprised two isolates with an average similarity of 82%. Thus MRSA could be divided into groups on the basis of inter-group dissimilarity and intra-group similarity of their immunoblot profiles.



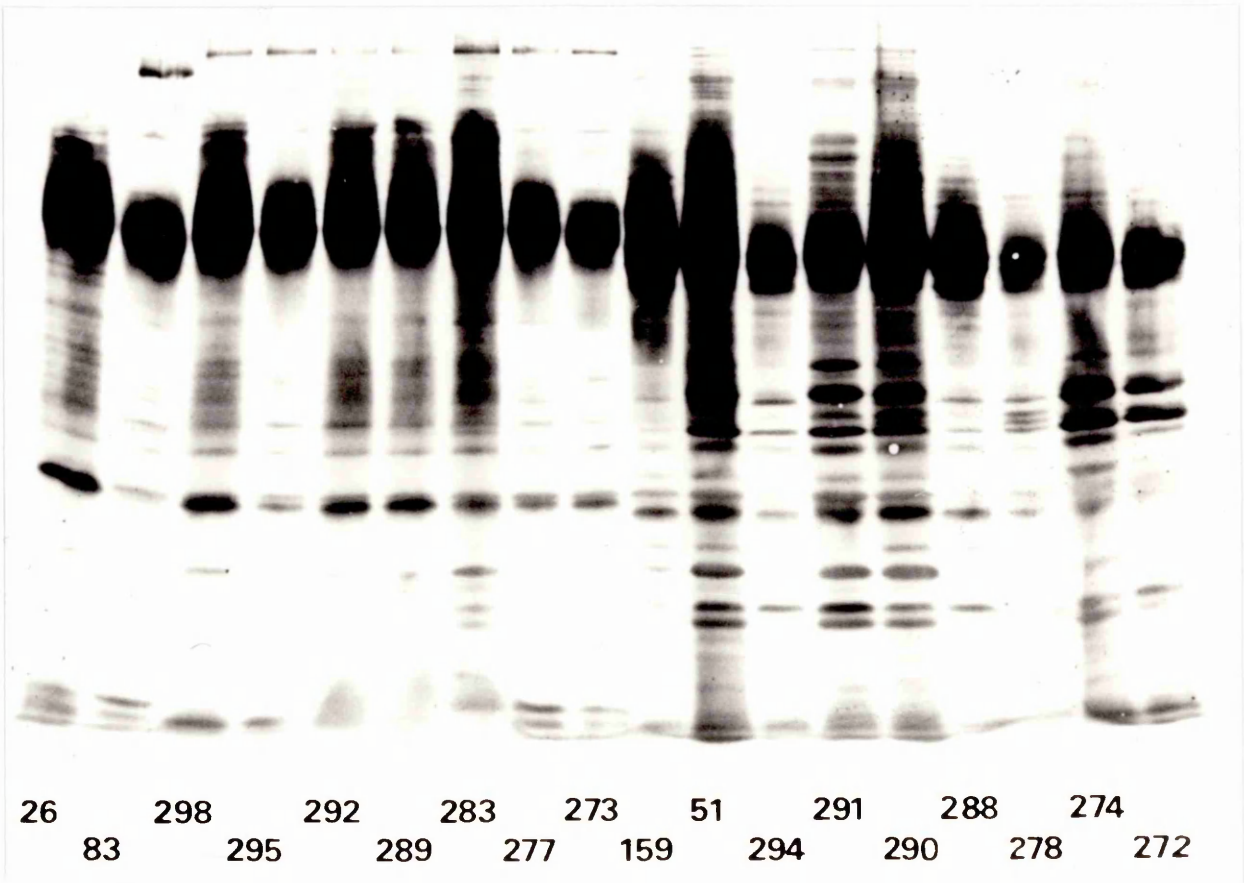


Figure 3. Typical immunoblots of MRSA culture supernates (numbers refer to isolates- add suffix 86).

PLASMID PROFILES

Plasmid-bearing isolates

Of the 163 MSSA strains, plasmids were detected in 42 % (21/50) of the BC isolates, 48 % (24/50) of the GP isolates and 41 % (26/63) of the RD isolates. The remainder of the BC, GP and RD isolates were found to be plasmid-free. In contrast, all of the 101 MRSA(1) and MRSA(2) isolates harboured plasmids. The number of plasmids harboured by each isolate is shown in Appendix 1. The minimum and maximum number of plasmids harboured by individual MSSA isolates was 0 and 2 respectively, whereas for the MRSA these figures were 1 and 4.

Distribution of plasmids

Table IX. Frequency distribution of plasmids in isolates from different sources

Plasmids per isolate	Number(%) of isolates with the given number of plasmids				
	BC (n=50)	GP (n=50)	RD (n=63)	MRSA(1) (n=53)	MRSA(2) (n=48)
0	29 (58)	26 (52)	37 (59)	0 (0)	0 (0)
1	19 (38)	22 (44)	22 (35)	6 (11)	6 (13)
2	2 (4)	2 (4)	4 (6)	44 (83)	41 (85)
3	0 (0)	0 (0)	0 (0)	2 (4)	1 (2)
4	0 (0)	0 (0)	0 (0)	1 (2)	0 (0)

The frequency distribution of plasmids in isolates from the different sources is shown in Table IX. The distributions observed in the BC, GP and RD collections did not differ significantly ($p > 0.5$). Similarly, the distributions found in the MRSA(1) and MRSA(2) groups were not significantly different ($p > 0.5$) (Figure 4). In view of the similarity in these distributions the results of the MSSA groups were combined for the purpose of comparison with the results of the MRSA collections which were also merged due to their similarity (Table X). This demonstrated a clear and significant difference in the plasmid distributions between the MSSA and MRSA groups ($p < 0.001$) (Figure 5).

Table X. Frequency distribution of plasmids in combined MSSA and MRSA isolates compared

Plasmids per isolate	Number(%) of isolates with the given number of plasmids	
	All MSSA (n=163)	All MRSA (n=101)
0	92 (56)	0 (0)
1	63 (39)	12 (12)
2	8 (5)	85 (84)
3	0 (0)	3 (3)
4	0 (0)	1 (1)

Plasmid sizes

The molecular sizes of the plasmids ranged from approximately 3 kb to 51 kb, and these could be divided into two

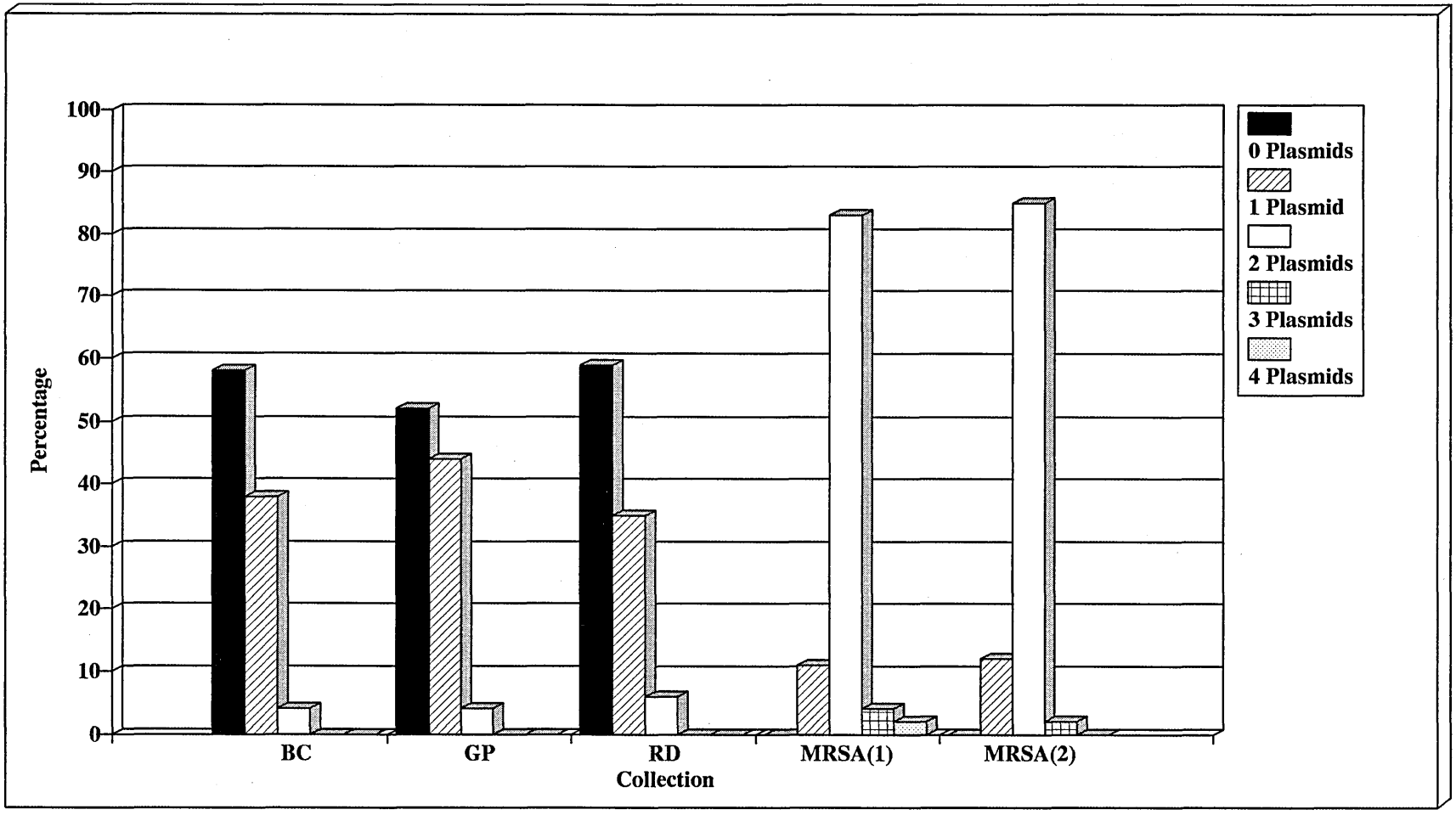


Figure 4. Frequency distribution of plasmids in isolates from different sources.

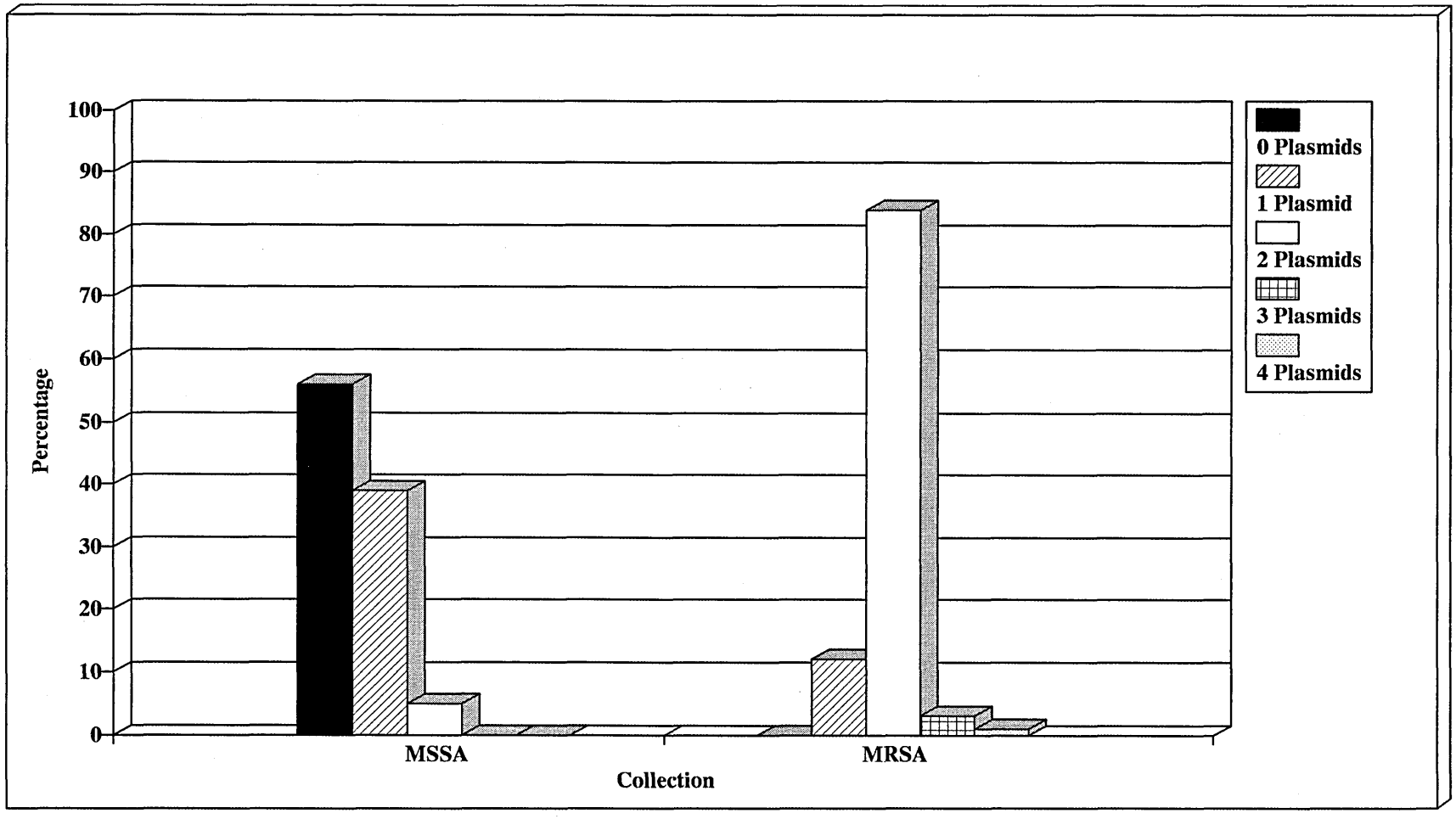


Figure 5. Comparison of frequency distributions of plasmids in MSSA and MRSA isolates.

groups; one of 15 kb to 51 kb, and one of 3 kb to 6 kb. Where an isolate was found to harbour a single plasmid, this plasmid was invariably of the larger size group: the small-size-group plasmids were never found alone. If an isolate harboured more than a single plasmid, a representative of each of the size groups was invariably present. Figures 6 and 7 show some typical MSSA and MRSA plasmid profiles which demonstrate these features. The thickened diffuse central band in each lane is derived from chromosomal DNA. Plasmid DNA bands appear as the much thinner and sharper bands present above and below the chromosomal band.

RESTRICTION ENZYME FRAGMENTATION PATTERNS

Number of REFPs

The 172 isolates (71 MSSA and 101 MRSA) which were found to harbour plasmids yielded a total of 56 unique restriction enzyme fragmentation patterns. The *Hae*III REFP of all the plasmid-bearing isolates are shown in Appendix 1. For all the isolates, the *Hae*III restriction patterns correlated well with those obtained by digestion with *Pst*I and *Alu*I. Typical REFPs are shown in Figures 8 and 9.

MRSA isolates

MRSA(1)

The MRSA(1) isolates yielded 11 different REFPs (Figure 10). Dice analysis of these patterns identified 2 major

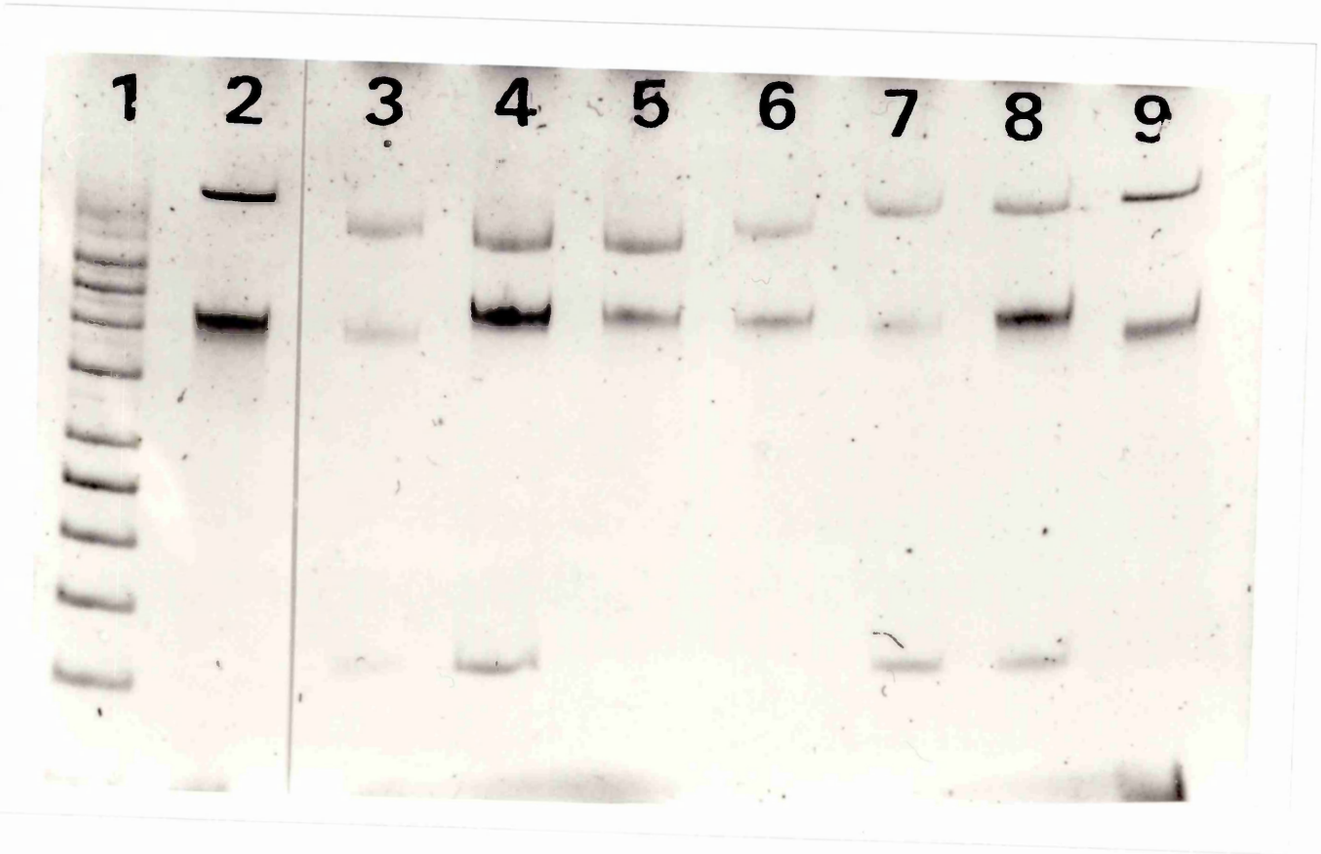


Figure 6. Typical *S. aureus* plasmid profiles. Lane 1-Supercoiled ladder standards; lanes 2 & 9-36 Md plasmid standards; lanes 3 & 4-two plasmid MSSA; lanes 5 & 6-two plasmid MSSA; lanes 7 & 8-two plasmid MRSA(1)(S) and MRSA(1)(R) respectively.

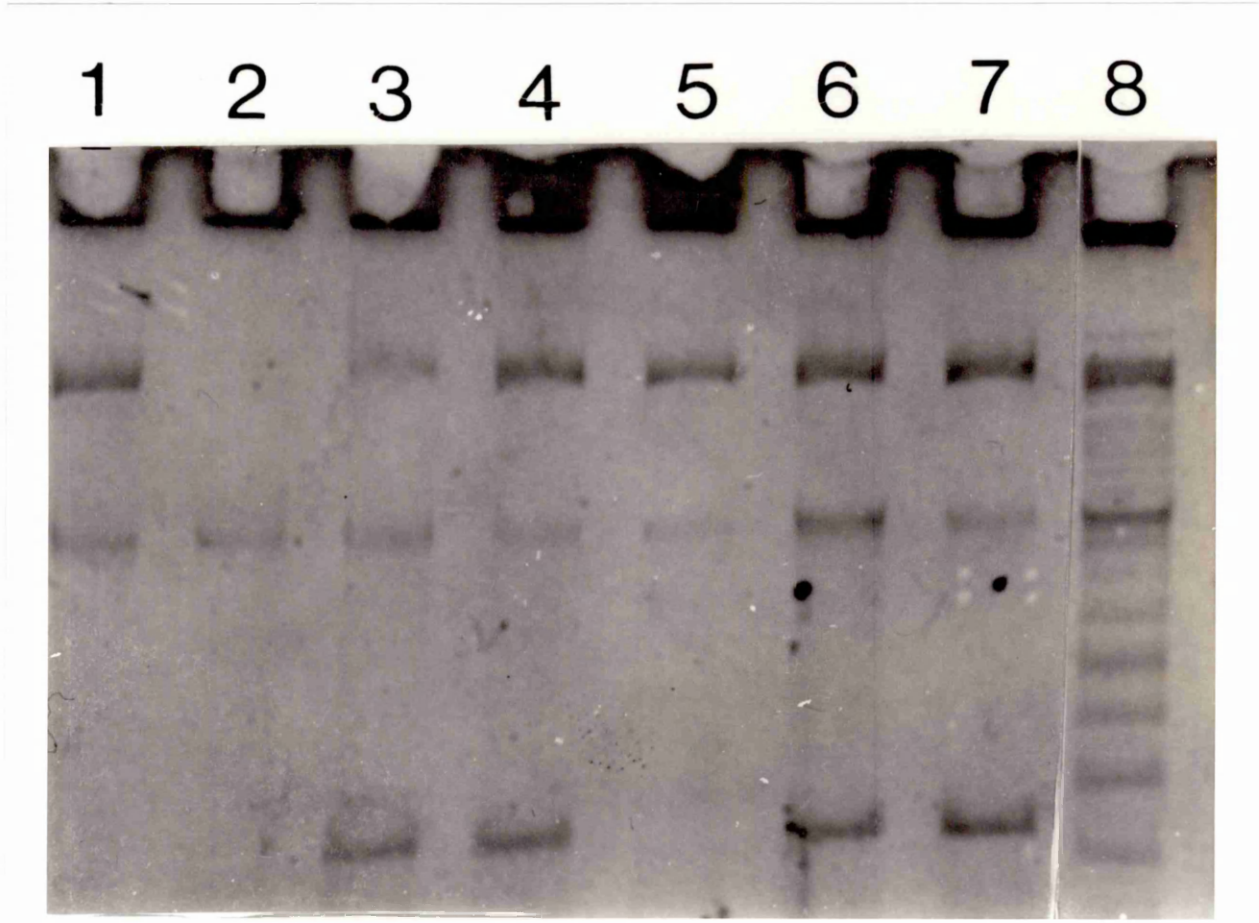


Figure 7. Typical *S. aureus* plasmid profiles. Lane 8-Supercoiled ladder standards; lane 2-plasmid-free MSSA; lanes 3 & 4-two plasmid MSSA; lanes 5 & 6-single & two plasmid MRSA(2)(S) respectively; lane 7-two plasmid MRSA(2)(R).

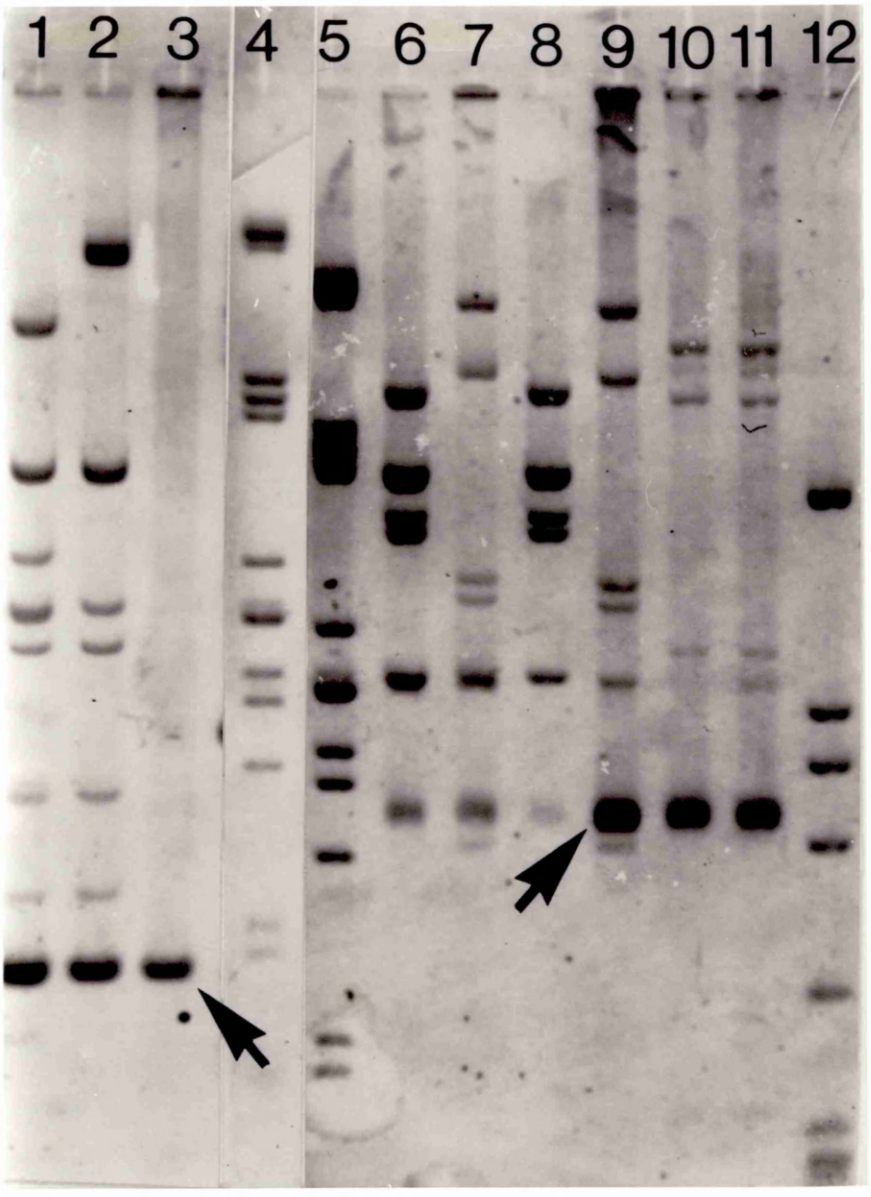


Figure 8. Typical *Hae*III restriction patterns of *S. aureus*. Lanes 1-4 and 5-12 are from two different gels; lanes 4 & 5-bacteriophage λ/*Pst*I fragments as calibration standards from each gel; lane 12-bacteriophage λ/*Hae*III fragments as calibration standards; lanes 1-3 -EMRSA; lanes 6 & 8- MRSA(1)(R); lanes 7 & 9-MRSA(1)(S); lanes 10 & 11-MSSA. Arrows indicate small multicopy plasmids present in MRSA and MSSA and their larger size with respect to those found in EMRSA.

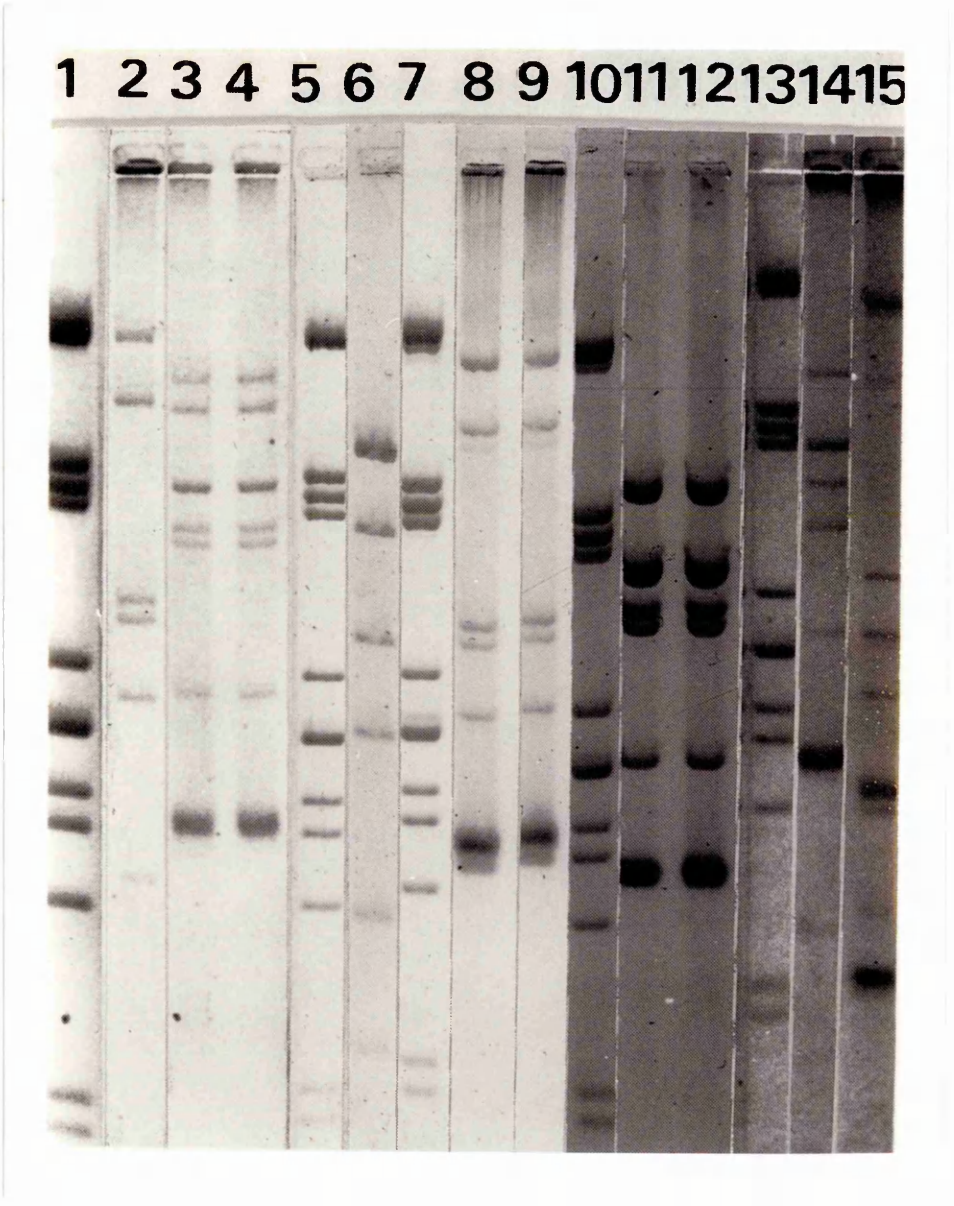


Figure 9. Typical *Hae*III restriction patterns of MRSA. Lanes 1-4, 5, 6, 7-9, 10-12, and 13-15 are from different gels. Lanes 1, 5, 7, 10 & 13-bacteriophage λ/*Pst*I fragments as calibration standards from each gel; lanes 2 & 6- single-plasmid MRSA(1)(S); lanes 3, 4 & 14- two-plasmid MRSA(1)(R); lane 15- three-plasmid MRSA(1)(S); lanes 8 & 9- two-plasmid MRSA(2)(S); lanes 11 & 12- two-plasmid MRSA(2)(R).

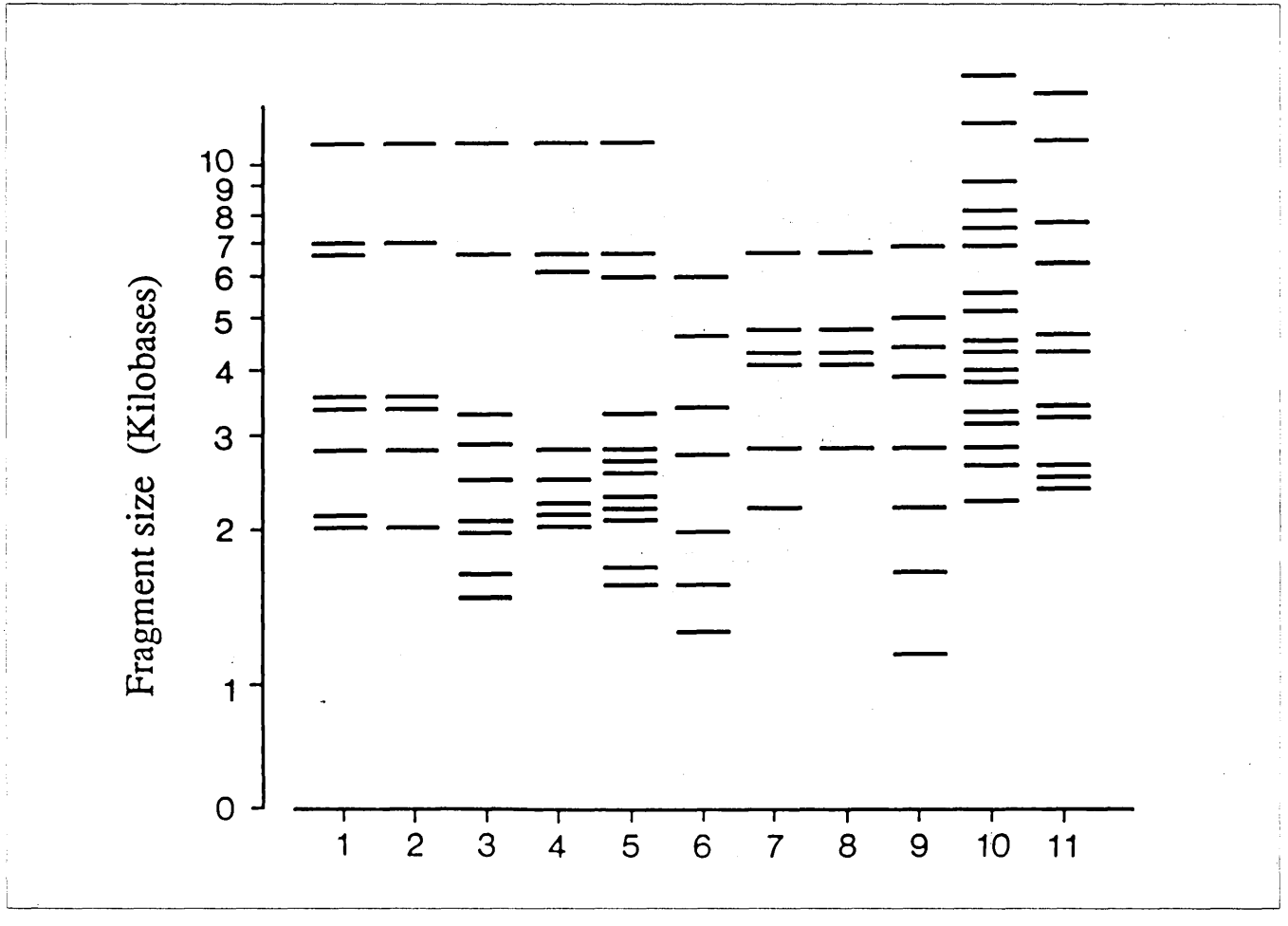


Figure 10. *Hae*III restriction enzyme fragmentation patterns of MRSA(1).

groups FP1 and FP2 which corresponded to the two predominant MRSA antibiograms described previously. The FP1 group comprised 23 isolates, with a mean similarity of 83 % (range 53 to 100 %). Ten of the FP1 isolates had identical REFPs (Pattern 1, Figure 10), and a further 7 had an REFP which differed from this predominant pattern only by the absence of 2 bands (Pattern 2, Figure 10). Two three-plasmid-bearing isolates (27986 and 28086) yielded a further REFP (Pattern 3, Figure 10), whilst the remainder of the FP1 group exhibited a variety of different REFPs with varying degrees of relatedness to the predominant FP1 pattern (Patterns 3, 4, 5, 6 and 11, Figure 10). All of the FP1 isolates were aminoglycoside-sensitive.

The FP2 group, which were aminoglycoside-resistant, comprised 28 isolates with a mean similarity of 97 % (range 71 to 100 %). Twenty-six of these isolates had identical REFPs (Pattern 7, Figure 10), and a further member of the FP2 group (30486) differed from this pattern by the absence of only a single band (Pattern 8, Figure 10). One further FP2 REFP was exhibited uniquely by isolate 05286 (Pattern 9, Figure 10).

Two isolates (08686 and 30586) did not group with either FP1 or FP2 or with each other (percentage similarity 16%), and were arbitrarily designated as FP3 and FP4 (Patterns 6 and 10 respectively, Figure 10), although they were observed to share fragments in common with both the predominant FP1 and FP2 patterns.

MRSA(2)

The 48 MRSA(2) isolates yielded 11 different REFPs (Figure 11), of which 4 were also found in the MRSA(1) collection (Patterns 1, 2, 9 and 10, Figure 11). Indeed, these common patterns included the predominant FP1 and FP2 patterns, and Dice analysis subdivided the MRSA(2) isolates into FP1 and FP2 groups as before.

The FP1 group comprised 19 isolates with a mean similarity of 87 % (range 42 to 100 %). Eight of the FP1 group yielded the predominant MRSA(1) FP1 pattern (Pattern 1, Figure 11), whilst a further 9 isolates yielded 5 REFPs which differed from this pattern only by the presence or absence of between 1 and 3 bands (Patterns 2, 3, 4, 5, and 6, Figure 11). Two further isolates (10687 and 10787) yielded 2 less closely related FP1 REFPs (Patterns 7 and 8 respectively, Figure 11).

The MRSA(2) FP2 group comprised 29 isolates with a mean similarity of 90 % (range 55 to 100 %). Twenty-six of these isolates yielded an REFP identical to the predominant MRSA(1) FP2 pattern (Pattern 9, Figure 11). Two further members of the FP2 group (13587 and 14587) differed from this pattern by the absence of a single band (Pattern 10, Figure 11), whilst the single remaining FP2 isolate (13187) differed by two bands (Pattern 11, Figure 11).

The FP1 and FP2 groups of the MRSA(2) collection corresponded as before with resistance and sensitivity to

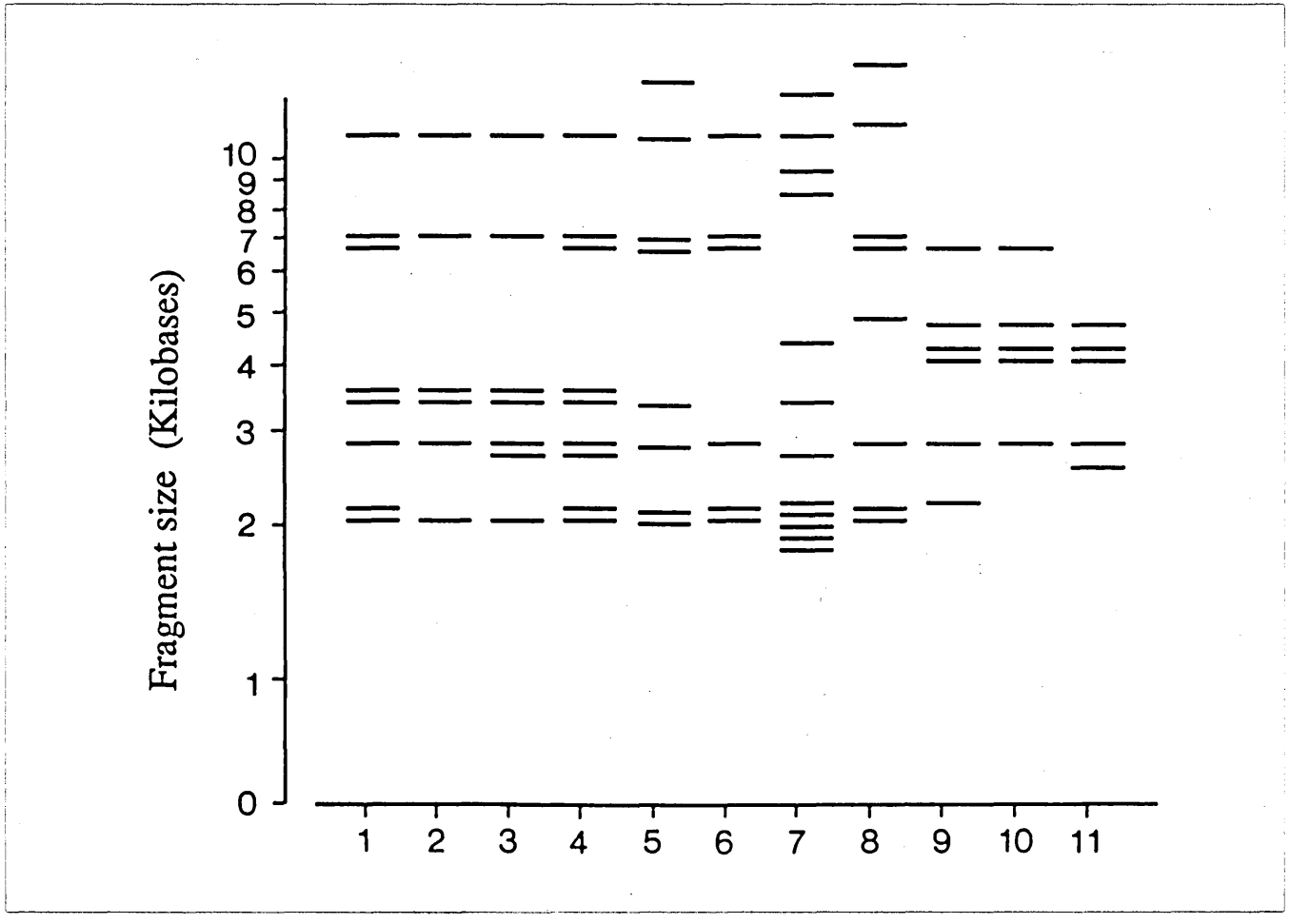


Figure 11. *Hae*III restriction enzyme fragmentation patterns of MRSA(2).

aminoglycoside antibiotics. Patterns 3-6 and 9-11 in Figure 10 and patterns 4-8 and 11 in Figure 11 were unique to the MRSA(1) and MRSA(2) collections respectively. No isolates corresponding to those designated FP3 and FP4 were found among the MRSA(2)

MSSA isolates

The MSSA isolates were in contrast much more heterogeneous, a total of 38 different REFPs having been identified amongst 71 plasmid-bearing isolates. However 6 different REFPs accounted for more than 40 % of plasmid-bearing MSSA isolates (Patterns 5, 6, 7, 8, 9 and 10, Figure 12). Dice analysis did not identify clear sub-groupings, although related REFPs were apparent among the patterns observed. Neither of the predominant MRSA REFP groups (FP1 and FP2) were similar to the MSSA REFPs (Figure 12). Therefore some identical REFPs, and hence plasmids, could be found in both community and hospital MSSA (Figure 12) but they were not present in MRSA.

Common features

Although common restriction patterns did not occur between MRSA and MSSA isolates, certain features of the REFPs were found in digests from both the MRSA and MSSA strains. A fragment of 2.7 kb after digestion with *Hae*III was present in all the REFPs from the MRSA isolates and in the patterns from more than 70% of the MSSA isolates (Figure 13).

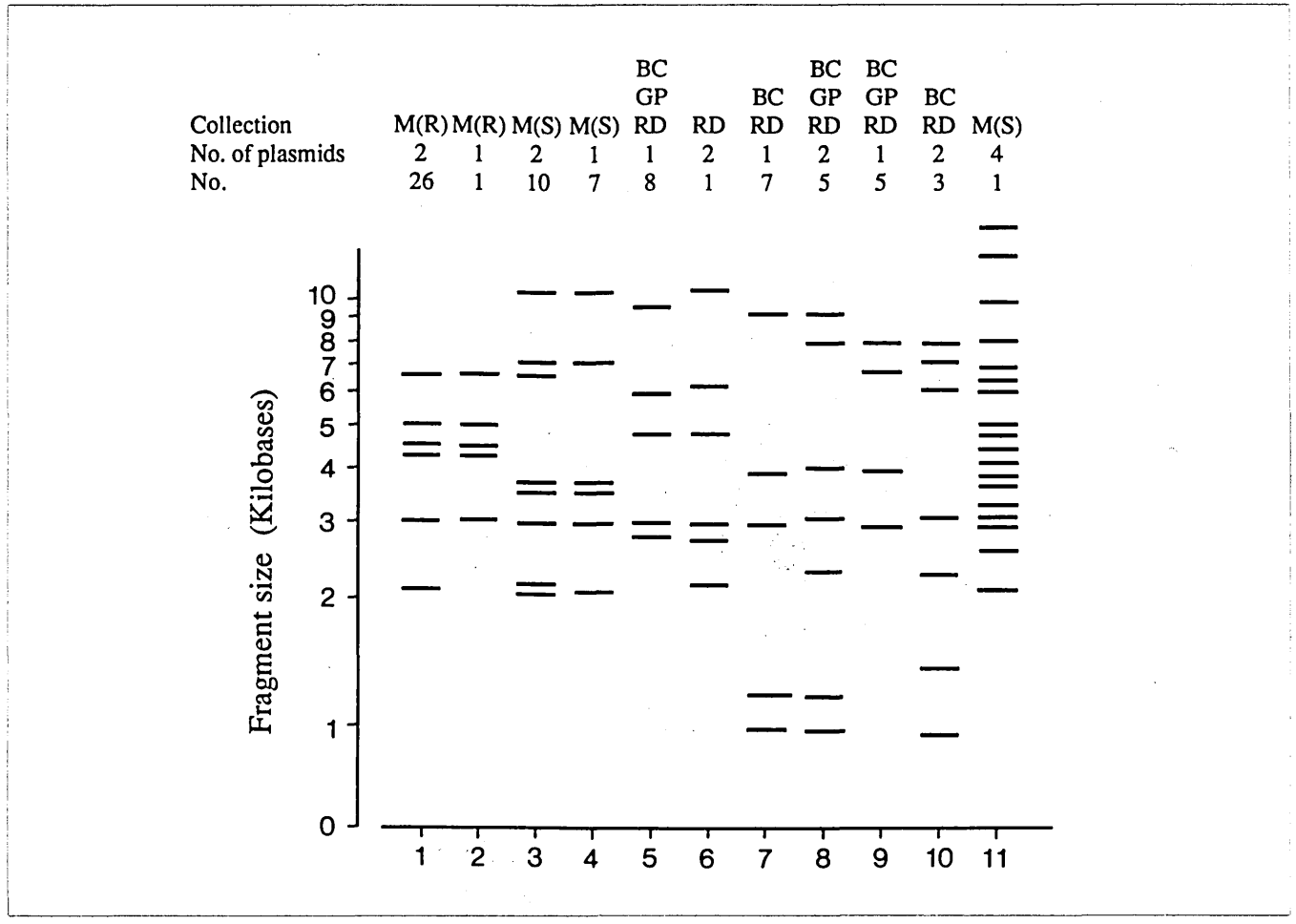


Figure 12. Examples of *Hae*III restriction patterns of *S. aureus* together withh their source collections, the number of plasmids harboured, and the number of strains showing identical fingerprints. M(R) and M(S) represent aminogycoside-resistant and -sensitive MRSA(1) isolates respectively.

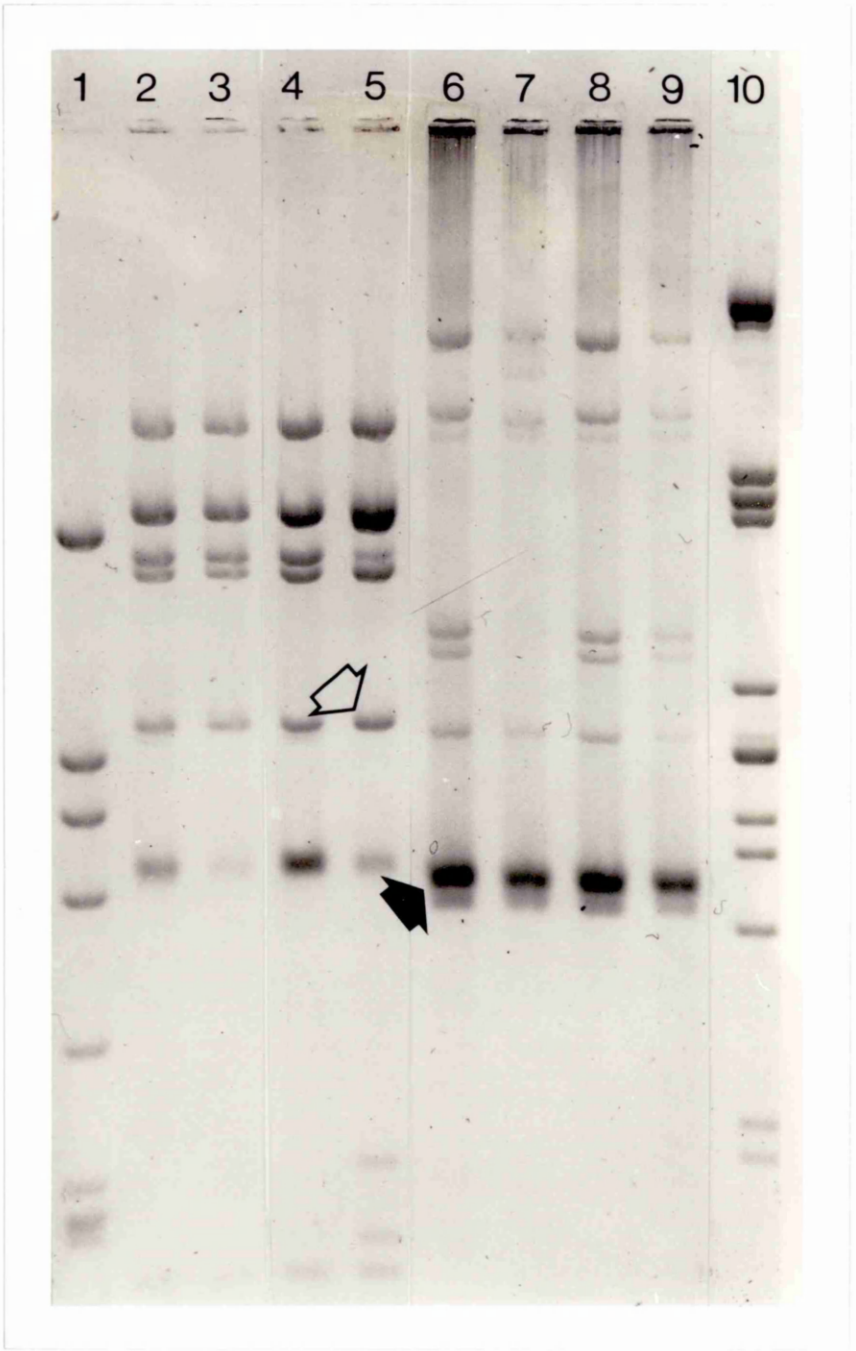


Figure 13. Typical *Hae*III restriction patterns of MRSA. Lane 1-bacteriophage λ/*Hae*III calibration standards; lane 10-bacteriophage λ/*Pst*I calibration standards; lanes 2 & 3 and lanes 4 & 5-MRSA(1)(R) and MRSA(2)(R) respectively; lanes 6 & 7 and lanes 8 & 9-MRSA(1)(S) and MRSA(2)(S) respectively. Open arrow indicates 2.7 kb *Hae*III restriction digest fragment present in all MRSA, and > 70 % of MSSA, REFPs. Closed arrow indicates small multicopy plasmids as found in MRSA and MSSA isolates harbouring more than one plasmid.

Small plasmids

Of particular interest were the small, multicopy plasmids present in those MRSA and MSSA isolates which harboured two or more plasmids. The relatively narrow size range of these plasmids revealed by plasmid profile gels has been mentioned already. These plasmids were not cleaved following digestion with any of the restriction enzymes *Pst*I, *Hae*III, *Ava*II, *Hin*dIII, *Sma*I, *Eco*RI or *Bsp*1286, and appeared in the REFPs as a single bright band (Figures 8 and 13). However, digestion with the enzyme *Alu*I of a variant of isolate 18586, which had spontaneously lost the larger of its two plasmids and hence contained only this small plasmid, produced five fragments (Figures 14a and 14b). The total molecular size of these fragments (2.6 kb) agreed well with the estimated size of the small plasmid determined from plasmid profile gels. The small plasmids in the other MRSA and MSSA isolates were similarly cleaved by *Alu*I. In all cases the same 5 fragments could be identified in the resulting REFPs, regardless of source. The observed differences between patterns 1 and 2, between 3 and 4, between 5 and 6, and between 7 and 8 in Figure 12 reflect the presence or absence of this plasmid.

Size heterogeneity

When the molecular weights of the fragments obtained by digestion are related to the plasmid profiles, it appears that the aminoglycoside-resistant MRSA isolates carried a highly conserved plasmid of around 18.3 kb, with a small number of molecular

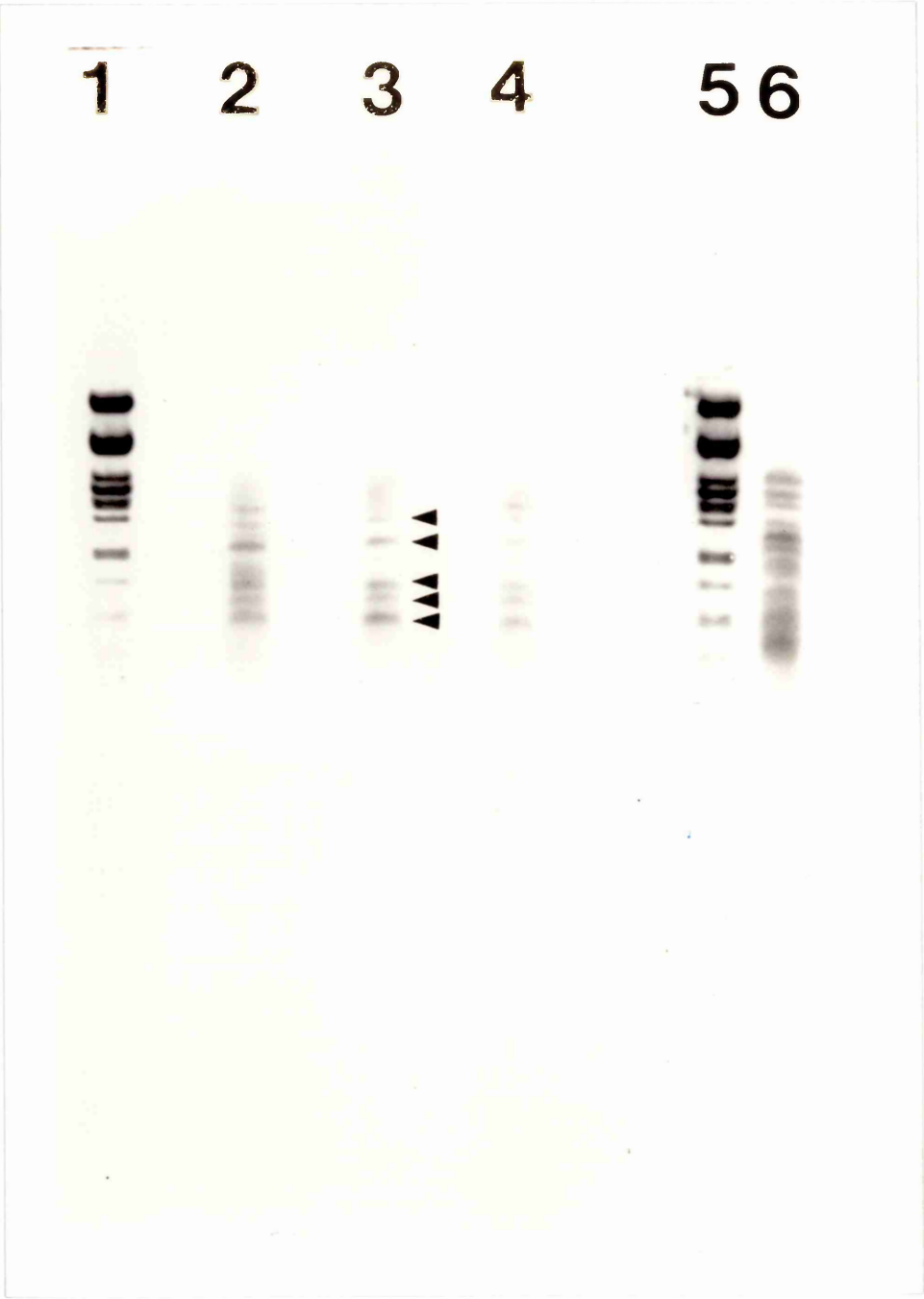


Figure 14a. *Alu*I restriction patterns of *S. aureus*. Lanes 1 & 5- bacteriophage λ/*Pst*I calibration standards; lane 6-bacteriophage λ/*Alu*I calibration standards; lane 2- 2 plasmid-bearing MRSA(1)(R); lane 4- 2 plasmid-bearing BC MSSA; lane 3- single small plasmid-bearing variant of isolate 18586. Photo of gel taken after running for 6 hours. Arrows indicate fragments derived from small plasmid.

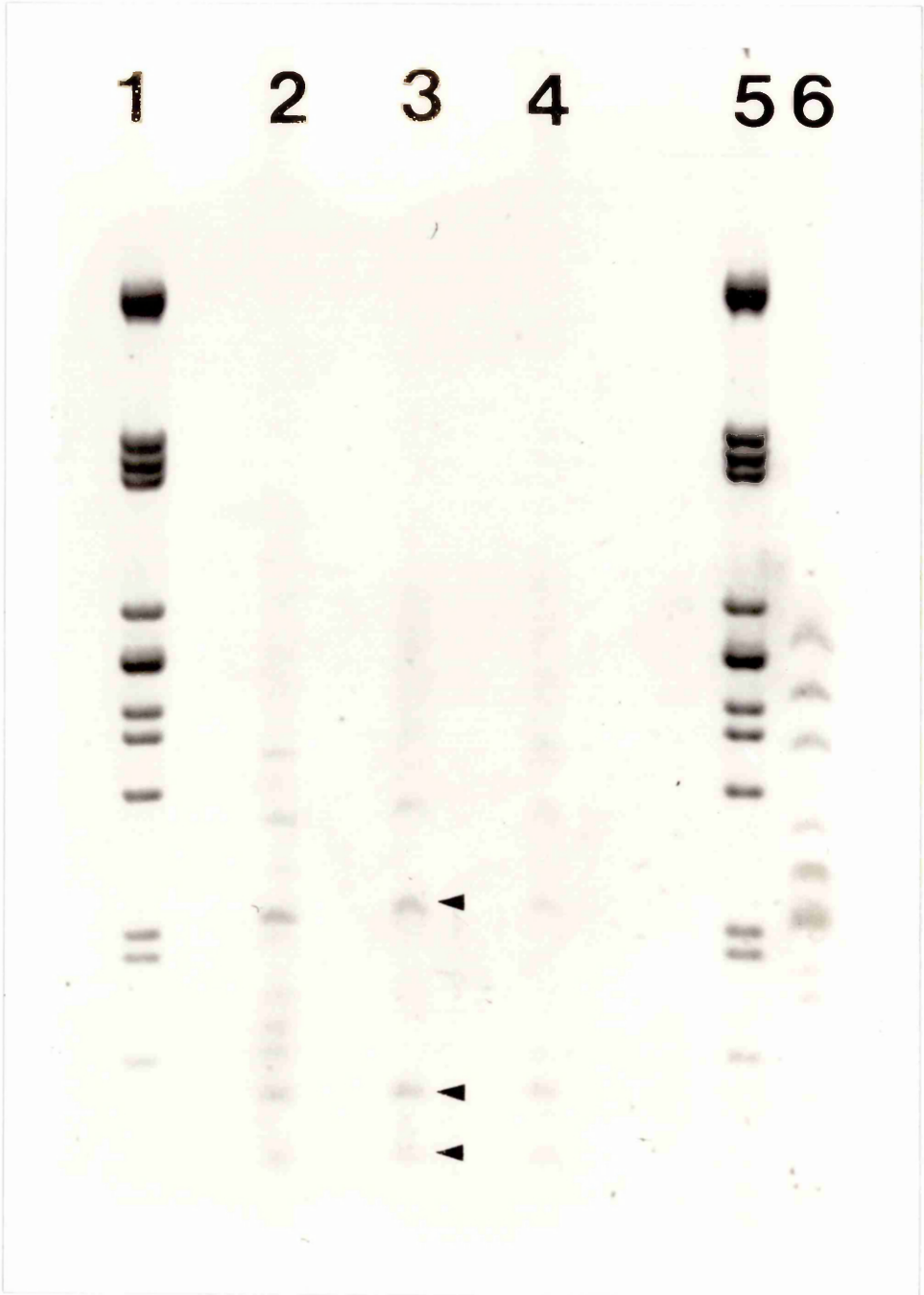


Figure 14b. Same gel as shown in Figure 14a. Photo of gel taken after running for 18 hours.

variants. The aminoglycoside-sensitive MRSA showed more variety, with their larger plasmids varying between 17 and 26 kb. However more than 70% of the aminoglycoside-sensitive MRSA carried the same 23.2 kb plasmid, and Dice analysis suggested that the majority of the other large plasmids in this group were molecular variants of this plasmid. The large plasmids in the MSSA were clearly more heterogeneous; more than 30 different plasmids with sizes ranging from 14 to 51 kb were identified. Those MRSA and MSSA isolates that carried more than one plasmid harboured a small plasmid of 2.6 kb. These plasmids were indistinguishable regardless of their origin. The commonest plasmids from the MSSA isolates, as judged by restriction patterns, were present in both hospital and community isolates Figure 12).

COMPARISON OF BIOTYPING, IMMUNOBLOTTING, REFPS AND PHAGE TYPING OF MRSA(1)

The relationship between the results of the various typing methods was assessed. Table XI shows the biotype, immunoblot group, REFP group and phage type (RTD) of each of the isolates. There was strong positive correlation between BLOT1 and FP1 groups, and between BLOT2 and FP2 groups ($p < 0.001$). In addition BLOT1/FP1 isolates were almost exclusively of biotypes A or C, whereas BLOT2/FP2 isolates were of biotypes B or D ($p < 0.001$) (Figure 15).

When phage typed, the BLOT1/FP1 strains were lysed by a selection of group 3 phages (predominantly 53, 83A and 85), the

Table XI. REFP group, immunoblot group, biotype and phage type (RTD) of MRSA(1)

	REFP group				Immunoblot group			Biotype				Phage Type (RTD)		
Isolate	FP1	FP2	FP3	FP4	BLOT1	BLOT2	BLOT3	BIOA	BIOB	BIOC	BIOD	Standard Set	Extended set	Supplementary for MRSA
2586	X				X			X				85	NA	616/617/622/625/630
28586	X						X	X				NT	90/932	617/622
27486	X				X			X				53/85	88A/90/932	616/617/622/626
27886	X				X			X				53/85	88A/90/932	616/617/622/626/630
27986	X				X			X				53/85	88A/90/932	617/622/626
8786	X				X			X				53/85	88A/90/932	616/617/622/626
28886	X				X			X				53/85	88A/90/932	616/617/622/626/630
27086	X				X			X				53/85	88A/90/932	616/617/622/626
29086	X				X			X				53/83A/85	88A/90/83C/932	616/617/622/626/630
15986	X				X			X				53/83A/85	88A/90/83C/932	616/617/622/626
27286	X				X					X		53/83A/85	90	NT
6986	X				X					X		53/75/83A/85	NA	617/623
28086	X				X					X		53/75/83A/85	88A/90/83C	617
29486	X				X					X		6/53/75/83A/85	88A/90/83C/932	617/623
29186	X				X					X		6/53/54/75/83A/85	88A/90/83C/932	617/622/630
5186	X				X				X			6/47/53/54/75/83A	88A/90/83C/932	616/617/622/623/626
1886	X				X			X				6/47/53/54/75/83A/85	NA	616/617/622/623/626
27686	X						X			X		53/75/83A/85	88A/90/83C/932	616/617/622/626
27386		X				X			X			NT	NT	NT
27786		X				X			X			NT	NT	NT
29886		X				X			X			NT	NT	NT
28786		X				X			X			NT	NT	NT
15686		X				X					X	NT	NT	NT
29286		X				X			X			NT	NT	NT
29586		X				X			X			NT	NT	NT
5286		X				X			X			NT	NA	617/622/630
27586		X				X					X	NT	NT	NT
28986		X				X					X	NT	NT	618/622
28386		X				X					X	NT	NT	NT
29386		X				X					X	NT	NT	NT
27186		X				X					X	NT	NT	NT
8086		X				X					X	NT	NA	NT
8186		X				X					X	NT	NA	NT
14986		X				X					X	NT	NA	NT
8286		X				X					X	NT	NA	NT
15386		X				X			X			NT	NA	NT
29686		X				X					X	NT	NT	NT
28686		X				X					X	NT	NT	620/630
30486		X			X						X	77/84	NA	617/618/622
2686		X				X					X	75/84	83C/932	NA
18086		X				X			X			NA	NA	NA
8386		X				X			X			NA	NA	NA
15786		X			X			X				53/85	NA	616/617/622/626
8686			X			X					X	NA	NA	NA
30586				X	X			X				29/52/52A/79/80/95/53/75/85	88A/90/83C/932	616/617/622/626/629/630

NA = Not Available; NT = Not Typable

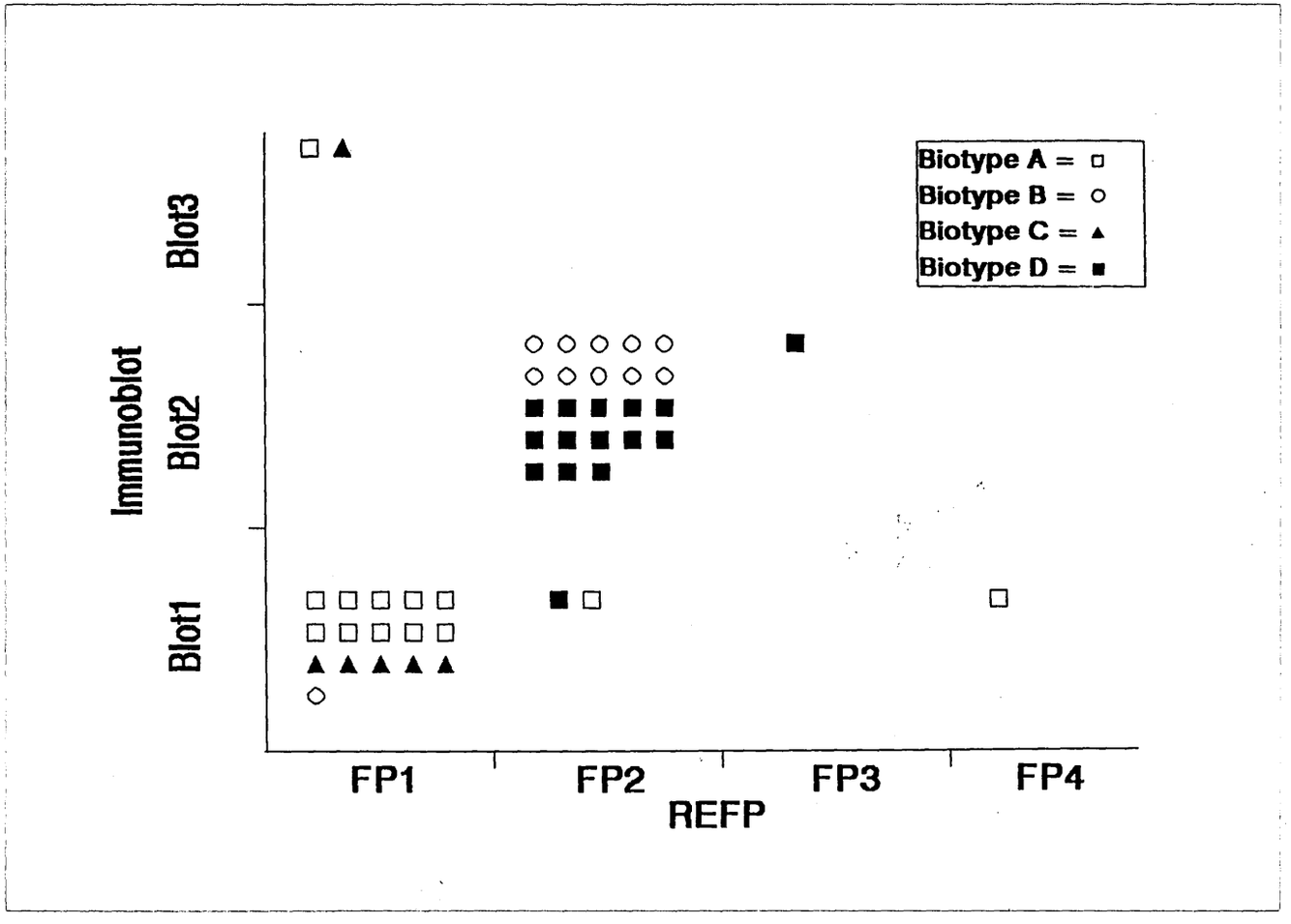


Figure 15. Correlation of immunoblot, REFP group and biotype of MRSA(1) isolates.

additional phages 88A, 90, 83C and 932 and the "new" set for typing MRSA (particularly 616, 617, 622 and 626). In contrast the BLOT2/FP2 strains were poorly typed at RTD, and even at RTD x 100 gave weak reactions to only a small number of phages (Phage typing data is presented in full in Appendix 2).

PATHOGENICITY FACTORS

Enterotoxin Production

Table XII and Figure 16 summarise the results of RPLA testing for enterotoxins A, B, C and D. The data are shown separately for the MRSA isolates designated aminoglycoside-sensitive or aminoglycoside-resistant, in order to demonstrate differences between them.

Table XII. Distribution of enterotoxin production in isolates from different sources

Enterotoxin	Number(%) of isolates producing enterotoxin				
	BC (n=48)	GP (n=46)	RD (n=58)	MRSA(S) (n=23)	MRSA(R) (n=26)
A	17 (35)	20 (43)	18 (31)	19 (83)	5 (19)
B	8 (17)	4 (9)	8 (14)	3 (13)	3 (12)
C	9 (19)	16 (35)	5 (9)	3 (13)	4 (15)
D	7 (15)	6 (13)	4 (7)	0 (0)	0 (0)

The results of tests for each of the individual enterotoxins are considered in detail below, followed by an analysis of the pattern

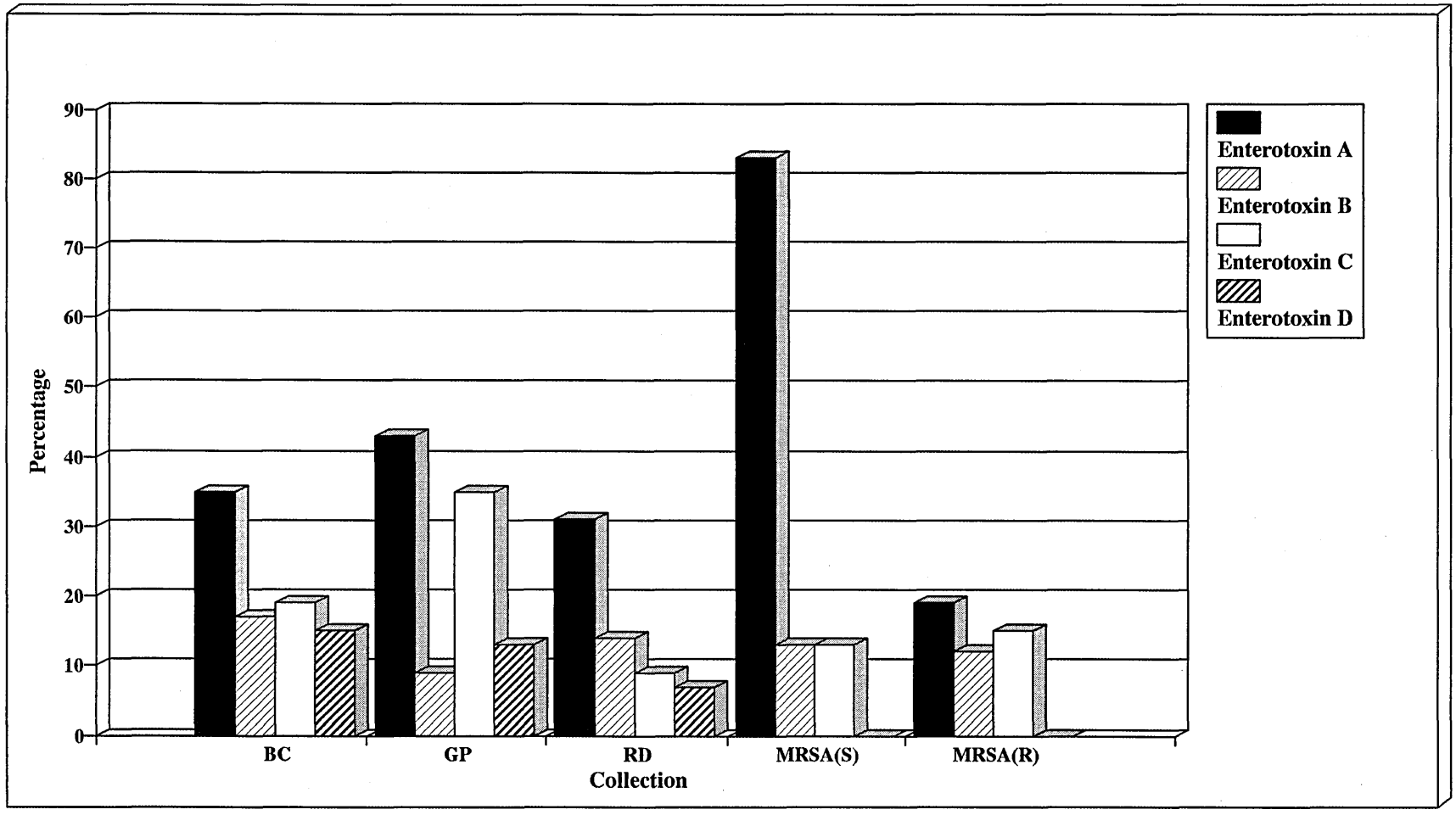


Figure 16. Results of RPLA tests for enterotoxins of *Staph. aureus*.

of enterotoxin production amongst the various collections. The enterotoxin results for individual isolates are presented in full in Appendix 3.

Distribution of individual enterotoxins

Enterotoxin A

When the number of enterotoxin A positive isolates in each of the three MSSA sub-groups was compared, there was no significant difference ($p > 0.5$), and for the purposes of comparison with the MRSA these results were combined. The relative number of enterotoxin A producing isolates did not differ significantly in the MSSA (55/152) and aminoglycoside-resistant MRSA (5/26) groups. However the aminoglycoside-sensitive MRSA differed markedly from the others with 83 % (19/23) isolates producing enterotoxin A ($p < 0.001$).

Enterotoxin B

The distribution of enterotoxin B positive isolates in each of the three MSSA sub-groups showed no significant difference ($p > 0.5$) and similarly, the distribution between aminoglycoside-sensitive and aminoglycoside-resistant MRSA collections was similar. When the combined MSSA results (20/152 enterotoxin B positive) were compared with the combined MRSA results (6/49 enterotoxin B positive) no significant difference was demonstrated ($p > 0.5$).

Enterotoxin C

No significant difference could be found in the distribution of enterotoxin C producers amongst the BC and RD MSSA collections. The proportion of positive isolates amongst these "hospital" MSSA strains (14/106) did not differ from that found in either the aminoglycoside-sensitive (3/23) or aminoglycoside-resistant (4/26) MRSA sub-groups ($p > 0.5$). However significantly more enterotoxin C positive isolates (16/46) were found amongst the GP isolates than in either the combined BC and RD MSSA (14/106), or the combined MRSA (7/49) ($p < 0.001$).

Enterotoxin D

There was no significant difference in the number of enterotoxin D positive isolates in any of the MSSA sub-groups ($p > 0.5$), which were again combined for the purpose of comparison with MRSA isolates. None of the MRSA isolates produced enterotoxin D. The difference between the number of enterotoxin D positive isolates in the combined MSSA (17/152) and MRSA (0/49) collections was significantly different ($p < 0.001$).

Distribution of enterotoxin-producing strains

Any isolate producing one or more enterotoxin(s) was defined an enterotoxin producer. Comparison of the relative numbers of enterotoxin producers in the various MSSA collections showed no significant difference ($p > 0.5$) (Table XIII).

Table XIII. Comparison of numbers of enterotoxin producers and non-producers amongst isolates from different sources

	Number(%) of isolates				
	BC (n=48)	GP (n=46)	RD (n=58)	MRSA(S) (n=23)	MRSA(R) (n=26)
Producers	29 (60)	29 (63)	30 (52)	20 (87)	7 (27)
Non-producers	19 (40)	17 (37)	28 (48)	3 (13)	19 (73)

There was a significant increase in the proportion of enterotoxin producers among the aminoglycoside-sensitive MRSA (20/23) when compared to the combined MSSA (88/152) ($p < 0.001$). However, significantly fewer enterotoxin producers were found among the aminoglycoside-resistant MRSA (7/26) than in either their aminoglycoside- or methicillin-sensitive counterparts ($p < 0.001$).

Table XIV. Comparison of multiple enterotoxin production by isolates from different sources

Toxins produced	Number(%) of isolates producing given number of enterotoxins				
	BC (n=48)	GP (n=46)	RD (n=58)	MRSA(S) (n=23)	MRSA(R) (n=26)
0	19 (39)	17 (37)	28 (48)	3 (13)	19 (73)
1	21 (44)	16 (35)	26 (45)	17 (74)	3 (12)
≥ 2	8 (17)	13 (28)	4 (7)	3 (13)	4 (15)

When the distribution of enterotoxins was examined in more detail by subdividing the isolates according to the production of 0, 1 or ≥ 2 enterotoxins (Table XIV), the BC and GP MSSA

distributions were found to be similar ($p > 0.5$). However, relatively fewer of the RD isolates produced ≥ 2 enterotoxins with a concomitant increase in the number of single enterotoxin producers, although this difference only achieved statistical significance when compared with the GP isolates ($p < 0.001$).

Seventeen of the 20 enterotoxin positive aminoglycoside-sensitive MRSA were single enterotoxin producers. All 17 of these isolates produced enterotoxin A. In contrast, although the aminoglycoside-resistant MRSA were predominantly non-enterotoxin producers, more than half of those which were enterotoxin positive produced more than one enterotoxin. Thus the pattern of enterotoxin production of both aminoglycoside-sensitive and aminoglycoside-resistant MRSA differed markedly from that of the MSSA ($p < 0.001$) but for different reasons; the former group because of the vast preponderance of single enterotoxin A producers, the latter group as a result of the large number of non-enterotoxin producers (Figure 17).

Haemolysin Production

Table XV and Figure 18 summarise the results of tests to detect the production of α-, β-, γ- and δ- haemolysins. In the interests of consistency with the presentation of results for enterotoxin production, the aminoglycoside-sensitive and the aminoglycoside-resistant MRSA isolates are again shown

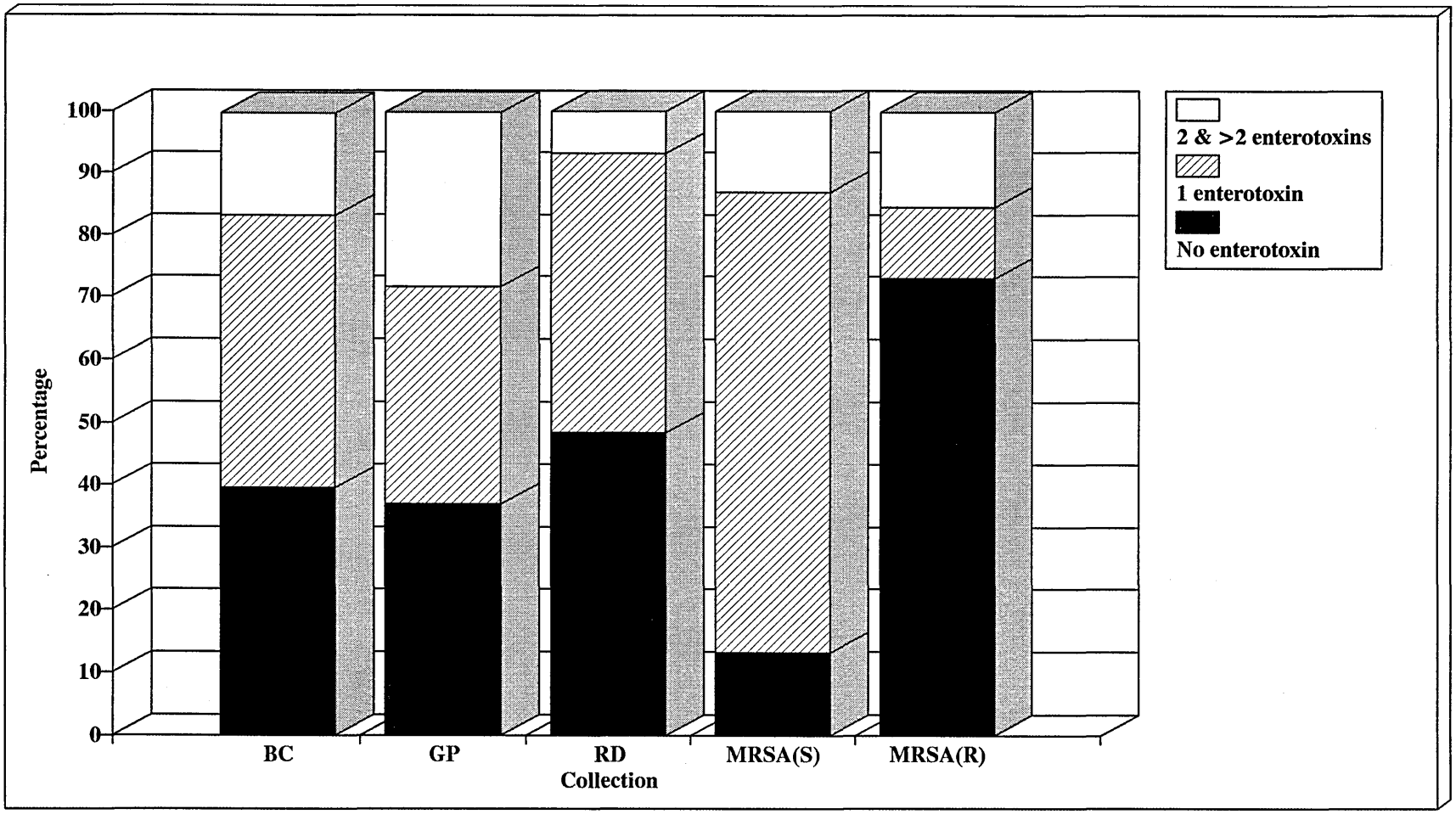


Figure 17. Distribution of multiple enterotoxin production by *Staph. aureus* from different sources.

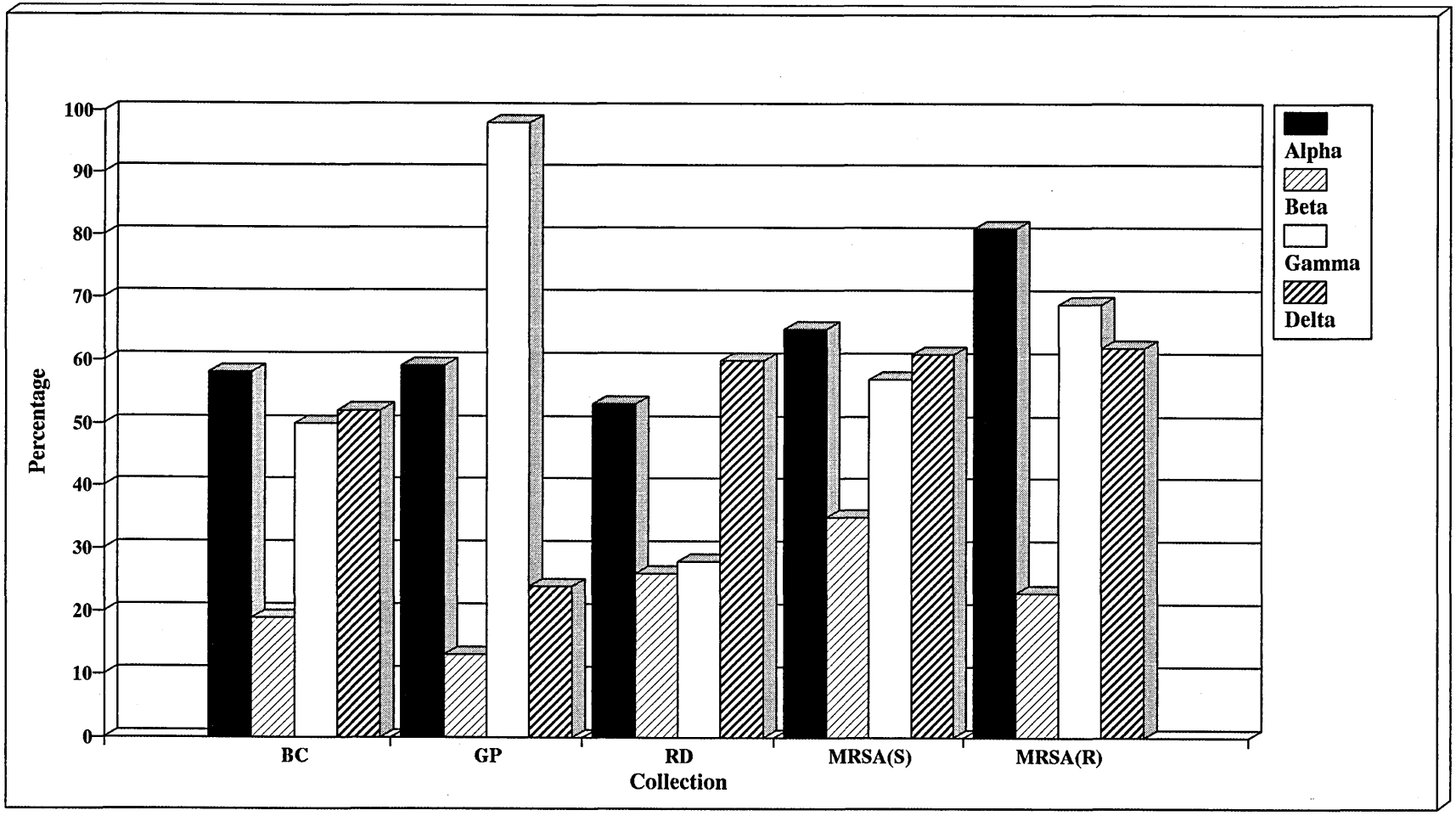


Figure 18. Results of tests for haemolysins of *Staph. aureus*.

separately, although there was no significant difference in the distribution of haemolysin production between these two groups.

Table XV. Distribution of haemolysin production in isolates from different sources

Haemolysin	Number(%) of isolates producing haemolysin				
	BC (n=48)	GP (n=46)	RD (n=58)	MRSA(S) (n=23)	MRSA(R) (n=26)
α	28 (58)	27 (59)	31 (53)	15 (65)	21 (81)
β	9 (19)	6 (13)	15 (26)	8 (35)	6 (23)
γ	24 (50)	45 (98)	16 (28)	13 (57)	18 (69)
δ	25 (52)	11 (24)	35 (60)	14 (61)	16 (62)

The results of tests for each of the individual haemolysins are considered in detail below, followed by an analysis of the distribution of haemolysin production among the various collections. The haemolysin results for individual isolates are presented in full in Appendix 3.

Distribution of individual haemolysins

α– haemolysin

Since the distribution of MSSA isolates which produced α–haemolysin did not differ between the BC, GP and RD sub-groups ($p > 0.5$), these were combined for comparison with the MRSA. Similarly the relative numbers of α– haemolysin producers among aminoglycoside-sensitive and aminoglycoside-resistant MRSA sub-

groups did not differ ($p > 0.5$). However, significantly more of the combined MRSA (36/49) produced α– haemolysin than the combined MSSA (86/152) ($p < 0.001$).

β– haemolysin

No significant difference was detected in the relative number of β– haemolysin producers amongst the various MSSA or MRSA sub-groups ($p > 0.5$). Similarly there was no difference in the relative combined total numbers of β– haemolysin producing MSSA (30/152) and MRSA (14/49) ($p > 0.5$).

γ– haemolysin

Considerably more GP isolates produced γ– haemolysin than strains from either the RD or BC collections ($p < 0.001$). A less marked, but nonetheless significant increase was also apparent in the number of γ– haemolysin producers among the BC compared with the RD MSSA ($p < 0.001$). The proportion of γ– haemolysin producers in the aminoglycoside-sensitive and aminoglycoside-resistant MRSA did not differ significantly ($p > 0.5$).

Sixty-three percent of MRSA (31/49), were γ- haemolysin producers, a number similar to that of the BC MSSA ($p > 0.5$), but somewhat higher and lower than the RD and GP MSSA respectively ($p < 0.001$).

δ– haemolysin

Significantly fewer GP than either BC or RD MSSA produced δ– haemolysin ($p < 0.001$). The BC and RD collections did not differ in this respect ($p > 0.5$). Similarly the aminoglycoside-sensitive and aminoglycoside-resistant MRSA did not differ significantly ($p > 0.5$). When the combined MRSA data (30/49) was compared with the combined BC and RD figures (60/106) as representing "hospital isolates" of MSSA there was no significant difference. However, it was clear that more MRSA produced δ– haemolysin than GP MSSA ($p < 0.001$).

Distribution of haemolysin-producing strains

Table XVI. Comparison of numbers of haemolysin producers and non-producers amongst isolates from different sources

	Number(%) of isolates				
	BC (n=48)	GP (n=46)	RD (n=58)	MRSA(S) (n=23)	MRSA(R) (n=26)
Producers	47 (98)	45 (98)	53 (91)	20 (87)	24 (92)
Non-producers	1 (2)	1 (2)	5 (9)	3 (13)	2 (8)

The isolates were divided into haemolysin producers and non-haemolysin producers; haemolysin producers were defined as any isolate which produced one or more haemolysin(s). Comparison of the relative numbers of haemolysin producers in the various MSSA collections showed no significant differences ($p > 0.5$) (Table XVI), and the MRSA collections were similarly

homogeneous. The relative total numbers of haemolysin producing MSSA (145/152) and MRSA (44/49) did not differ significantly ($p > 0.5$).

When the number of MSSA isolates in each of the BC, GP and RD sub-groups which produced < 2, 2 or ≥ 3 haemolysins was compared, no significant difference could be demonstrated (Table XVII) ($p > 0.5$). Similarly, there was no significant difference between the aminoglycoside-resistant and aminoglycoside-sensitive MRSA sub-groups ($p > 0.5$)[3]. When the distributions of numbers of haemolysins produced by MSSA and MRSA were compared (Figure 19), these differed, with the MRSA producing significantly more haemolysins ($p < 0.001$).

Table XVII. Comparison of multiple haemolysin production by isolates from different sources

Haemolysins produced	Number of isolates producing given number of haemolysins				
	BC (n=48)	GP (n=46)	RD (n=58)	MRSA(S) (n=23)	MRSA(R) (n=26)
< 2	21 (44)	14 (30)	23 (40)	4 (17)	4 (15)
2	17 (35)	21 (46)	26 (45)	10 (44)	8 (31)
≥ 3	10 (21)	11 (24)	9 (15)	9 (39)	14 (54)

[3]Due to the small numbers of MRSA isolates which produced less than two haemolysins, and in order to facilitate a valid statistical comparison, isolates producing 2 haemolysins were combined with those producing < 2 haemolysins.

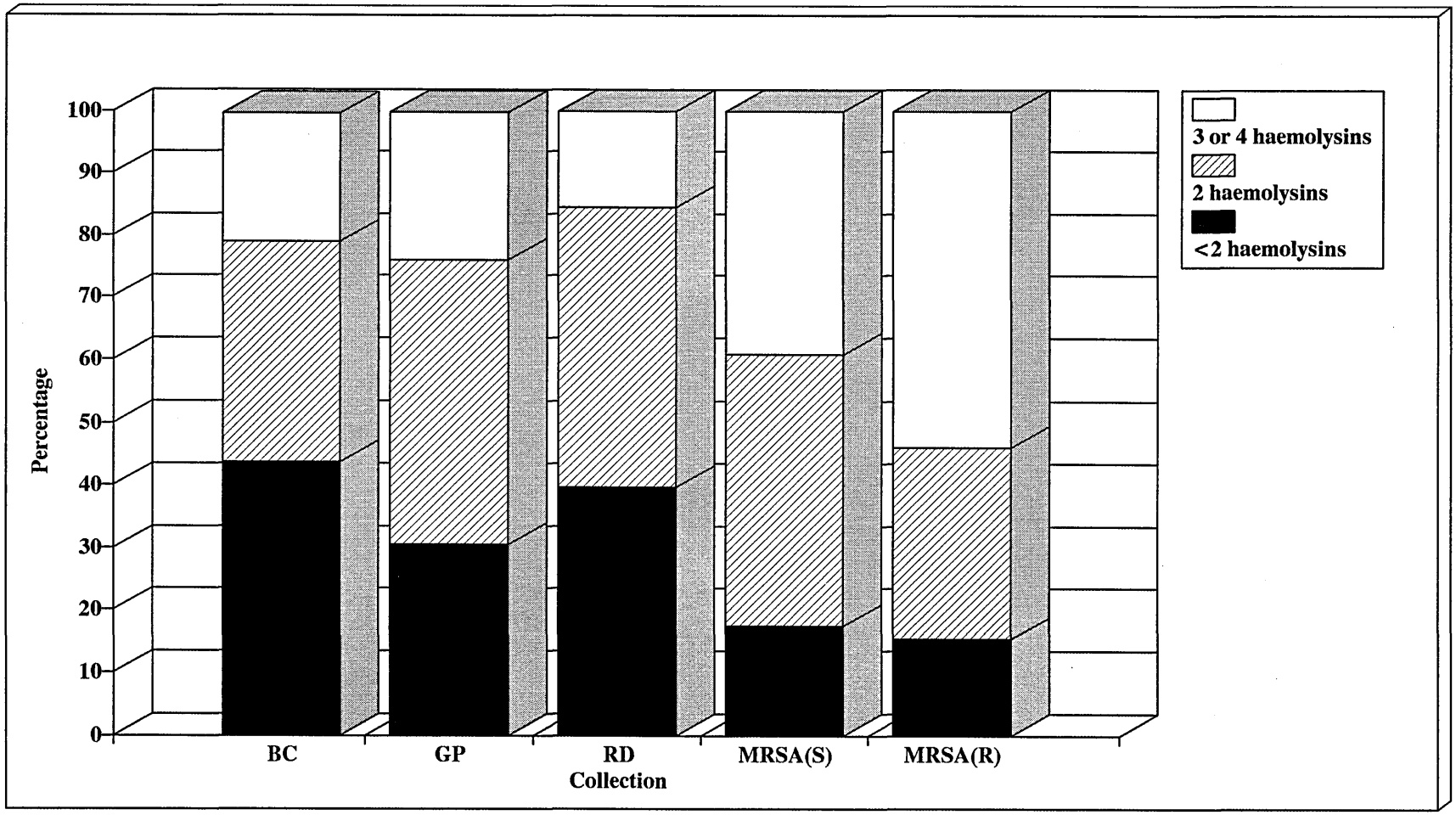


Figure 19. Distribution of multiple haemolysin production by *Staph. aureus* from different sources.

CHAPTER 4

Discussion

ANTIBIOTIC RESISTANCE

The antibiotic-sensitivity test results with MRSA re-emphasise that methicillin-resistant strains tend to be multiply resistant. Two antibiograms accounted for 94 % of all MRSA isolates, differing in the presence or absence of resistance to the aminoglycosides gentamicin, kanamycin and streptomycin, in addition to a "core" resistance to methicillin, penicillin, erythromycin and tetracycline present in all strains. These two sub-groups determined by aminoglycoside sensitivity were similar to the sub-groups defined by other phenotypic and genotypic markers.

The results of the sensitivity tests with MSSA isolates were unremarkable and in keeping with expectations for this population.

One of the more interesting results of sensitivity testing was the demonstration of mupirocin resistance in one of the MRSA isolates (Baird and Coia, 1987). This strain was isolated before the introduction of mupirocin into clinical practice and was probably unrelated to prior use of this agent. Thus it demonstrates the potential for antimicrobial resistance to emerge by the selection of pre-existing mutants.

PREVALENCE AND DISTRIBUTION OF PLASMIDS IN MSSA AND MRSA

The plasmids harboured by MSSA and MRSA differed in their prevalence, number and distribution. The most striking initial observation was that all MRSA harboured plasmids. This was in

sharp contrast to the MSSA, in which more than half of the isolates were found to be plasmid-free. Although plasmid-free isolates of MRSA have been described by Gelmi *et al* (1987), most other studies report that the majority of MRSA do harbour plasmids.

The frequency distribution of plasmids in MRSA and MSSA isolates was markedly different. However, it was interesting to compare these distributions with those predicted by the model developed by Platt (1987) to analyse the distribution of plasmids amongst representative collections of enterobacteria. This model was based on previous observations that plasmid-free strains contributed disproportionately to the overall frequency distribution of plasmids in such collections. Therefore, in this model a modified Poisson distribution conditional upon $n \neq 0$ was used to predict the frequency distributions of plasmids in plasmid-containing strains. Such predictions agreed well with the distributions observed in this study.

The distributions predicted by the application of this model to the MSSA and MRSA collections are shown in Table XVIII. Amongst MSSA isolates, the observed frequency distribution of plasmids did not differ from the model prediction. What does this tell us about the MSSA? First, in line with the arguments advanced by Platt and co-workers, this suggests that the majority of plasmid-free MSSA isolates are poor plasmid recipients (Platt *et al*, 1984; Platt, 1987). Second, it supports the related hypothesis that the

accumulation of plasmids by Gram-negative and Gram-positive genera may be subject to the same variables.

Table XVIII. Comparison of observed plasmid distributions in MSSA and MRSA with distributions predicted by a modified Poisson model

Plasmids per isolate	Number of isolates with given no. of plasmids			
	MSSA		MRSA	
	observed	predicted	observed	predicted
1	62	63	12	47
2	10	8	85	32
3	0	0	3	15
4	0	0	1	6

The observed frequency distribution of plasmids from MRSA isolates differed significantly ($p < 0.001$) from that predicted by the model. The distributions were somewhat skewed, with a marked preponderance of isolates which harboured 2 plasmids. These findings were consistent with the hypothesis that the MRSA isolates represent dissemination of a limited number of clones within Glasgow Royal Infirmary.

COMPARISON OF MSSA AND MRSA REFPS

The REFPs from the MRSA isolates were markedly less diverse than amongst the MSSA. As has been shown (p. 79), application of Dice analysis to the observed *Hae*III patterns demonstrated two major groups of MRSA, FP1 and FP2. The majority of isolates in each of these groups yielded an identical

"prototype" pattern, with other isolates in the groups exhibiting greater or lesser degrees of variation from these. This variety arose from two distinct sources. First, the presence or absence of the uncut small plasmids which yielded characteristic changes to the REFP. The resultant alteration to the REFPs was thus the result of a discrete change in number of plasmids harboured. The second component to REFP variability in each of the major MRSA REFP groups was molecular rearrangements in the larger MRSA plasmids. This phenomenon produced either loss of DNA fragments from the respective prototype pattern and/or the presence of extra DNA fragments additional to the group prototype.

It has been noted that the large plasmids in aminoglycoside-resistant MRSA were less variable than in their aminoglycoside-sensitive counterparts. It is interesting to speculate whether this relative lack of diversity reflected their somewhat more circumscribed epidemiological distribution within the hospital; aminoglycoside-resistant MRSA are largely, although not exclusively, isolated from the Burns and related units.

Comparison of the MRSA(1) and MRSA(2) isolates was largely unremarkable. The same basic patterns were observed, and there was no evidence amongst the MRSA(2) of either radical rearrangement of the plasmids found in MRSA(1) or the presence of novel large plasmid species. Small plasmids were homogeneous as described already.

Thus, the majority of MRSA harboured two plasmids. These plasmids comprised a small 2.6 kb species common to all MRSA isolates, and variants of a larger plasmid of either 18.3 kb or 23.2 kb in aminoglycoside-resistant and aminoglycoside-sensitive strains respectively. The few MRSA isolates which harboured more than 2 plasmids shared variable numbers of common fragments with the prototype MRSA REFPs, although it is not surprising that their Dice co-efficients of similarity were lower as a result of the extra DNA fragments yielded by the digestion of the additional plasmids.

The MSSA REFPs were more diverse than the MRSA. The combined REFP and profile data were consistent with them harbouring more than 30 plasmids in the range 14.5 - 50.7 kb. It is clear that this was an important "third factor" in the generation of diversity, additional to the two factors outlined above for the MRSA sub-groups.

Common REFPs and plasmids were found both within and between the various MSSA sub-groups. It seems likely that this reflected interchange of strains although, of course, it may have been the result of exchange of plasmids. MRSA REFPs were not found amongst the MSSA, although some DNA fragments, most notably the 2.7 kb *Hae*III fragments, were shared between the two groups.

An issue which is debated frequently when considering epidemiological data based on plasmid studies is the relative instability of such elements. In the course of this study a number of

isolates were re-examined after prolonged storage (> 12 months) and repeated sub-culture (20-30 passages). It was reassuring that the results of these experiments showed that there was good stability *in-vitro*. A more interesting observation when comparing the MRSA(1) and MRSA(2) collections was that this stability could be demonstrated *in-vivo* because these isolates had been collected over an extended time period. This stability has not been found in preliminary studies of *Staphylococcus epidermidis* which suggests that whereas these plasmids may be stable *in-vitro* major molecular re-arrangements may take place *in-vivo* within a very short time (J. Coia, unpublished data). This difference between two staphylococcal species highlights the fact that the application of plasmid data to epidemiological questions requires the establishment of a proper interpretative framework on a species by species basis. Indeed such considerations of applicability and stability of typing characteristics should be explored rigourously for any novel or, for that matter, existing technology.

ORIGINS OF MRSA WITHIN GRI

When taken together the plasmid distribution data and the limited REFP diversity of MRSA argue strongly in favour of clonal dissemination within the hospital, but the question of the original source of the MRSA remains unanswered. One possibility is, that it was exogenous with subsequent spread favoured by selection pressure. An example of such a process has been elegantly documented in Dublin hospitals (Carroll *et al*, 1989). Such a model

is attractive in explaining the origin and evolution of the current MRSA epidemic. However, the large number of geographically distinct isolations of MRSA organisms which behave very differently in terms of microbiological and clinical characteristics, within a relatively short space of time raises the possibility that MRSA may have evolved locally from endogenous MSSA strains.

This alternative hypothesis is supported by some of the data presented here, particularly the finding that the small multicopy plasmids are identical in both MSSA and MRSA. Many of the isolates of MRSA from patients in different hospitals in England, and which have been described as epidemic MRSA (EMRSA) (Cookson *et al*, 1986), carry small plasmids similar in size to those of the local MRSA as described here. However, their REFPs (Figure 8, p. 78) differed markedly from those of local MRSA. In addition, the small plasmids harboured by MRSA and MSSA from this hospital are indistinguishable, but differ from small plasmids of EMRSA. These facts are all in favour of the local evolution *de novo* of local MRSA from methicillin-sensitive strains.

The most likely progenitor strain for such a process would be a two plasmid-bearing MSSA. However, such isolates were rare amongst MSSA strains, and the small number of such isolates studied in this collection does not preclude the existence of such progenitors, which would be expected to yield REFPs more similar to their methicillin-resistant counterparts.

The two hypotheses for the origin of MRSA are not mutually exclusive; both processes may have occurred in different locations at different times. Regardless of source, exogenous or endogenous, the same balance of positive and negative selection pressures would influence subsequent spread and evolution within an institution. A further finding which requires consideration is the relationship between the two major MRSA REFP groups; either there was a single MRSA clone which diverged early to give rise to these distinct patterns or two separate events established the separate clones. It is not possible to give a definitive answer to this question, but the marked differences in REFP, together with the differences demonstrated by the other typing methods, all of which must have developed during the relatively short period during which MRSA have been present in the hospital, favour the latter explanation. The alternative possibility requires the very rapid divergence of a single clone, which seems less likely given the very similar environments in which such a process would have to have occurred.

COMPARISON OF CHARACTERISATION OF MRSA BY BIOTYPING, IMMUNOBLOTTING AND REFPS

One of the aims of this study was to explore to what degree the information derived from different methods could usefully be combined in an epidemiological context. It was not intended to establish a definitive method for the typing of MRSA. Clearly each of the methods described has associated strengths and weaknesses. The methods adopted by any individual centre to monitor the spread

of MRSA will ultimately be determined by local expertise and experience, available facilities and prevailing resource constraints.

Biotyping is attractive, because it is simple, quick and reproducible, and can easily be incorporated into the daily bench routine of a diagnostic clinical microbiology laboratory. However, in the scheme described here, due to the limited number of characteristics tested, strain discrimination was limited. It may be that the incorporation of additional tests, such as those for haemolysin or enterotoxin production, would increase discrimination. However, this would detract from the major virtues of low cost and simplicity. A further consideration concerns the wider applicability of the scheme utilised here. Although this biotyping method was very useful in epidemiological monitoring within GRI and its associated hospitals, such a simple scheme on its own may not have the same value in the analysis of a broader-based collection of *Staph. aureus* strains.

Phage typing is standardised, reproducible and universally acknowledged as a definitive method for the investigation of outbreaks of infection with *Staph. aureus* (Parker, 1983). It is rapid to perform and apparently relatively inexpensive to perform, but there are hidden costs in the maintenance of stocks of phages. In addition, the best and most reproducible results are those from reference centres which may be geographically remote from the laboratories in which the organisms have been isolated. However, the most important drawback with respect to MRSA is that the

organisms may be poorly lysed by phage, or not at all. This was a problem encountered with significant numbers of the isolates studied here.

Immunoblots, either of whole-cell or exported proteins, have been used increasingly in the epidemiological investigation of outbreaks of infection (Lee *et al*, 1986; Burnie *et al*, 1988; McKay *et al*, 1988). The characteristic profiles produced provide a rapid, reproducible and sensitive method for characterisation of a range of organisms using the same apparatus and techniques. Furthermore, all the antigenic polypeptides of each isolate can potentially contribute to the immunoblot profile, rather than relying on the expression of a limited number of biological, biochemical or morphological characteristics. However capital costs are undoubtedly high, and specialist technical expertise is required. If immunoblotting was to be employed on a wider scale, with analyses performed at a number of centres, standardisation of reagents and the methods used to document results would be required to allow inter-centre comparability of the data generated (Hawkey, 1987).

REFP analysis shared many common advantages and disadvantages with immunoblotting. This technology removes completely the requirement for phenotypic expression to facilitate typing, and indeed variations in the entire genome of the organism, including non-coding regions, are amenable to analysis. Since all diversity ultimately derives from alterations to an organisms DNA, this is the most fundamental level at which to study such variation.

Disregarding cost and expertise, and these considerations should become less important with advances in technology and automation, the major problem that remains is how to compare data from different laboratories. Many of the interpretative problems could be solved by the adoption of standardised restriction enzyme digestion strategies (Platt *et al*, 1986).

Perhaps the most interesting finding in this study was the general agreement between the results generated by these disparate typing methods. Not only did the techniques mutually validate one another, but each method, in a more or less direct fashion, demonstrated differences arising from a limited number of underlying genotypes. Clearly there were two major groups of isolates:- FP1 / Biotype (A+C) / BLOT1 and FP2 / Biotype (B+D) / BLOT2 (Figure 15). Moreover, most strains in the former group produced enterotoxin, whereas strains in the latter tended not to do so. It was not possible to conclude if the small number of isolates in the BLOT3 and FP3/4 groups represented other distinct subgroups, and the investigation of further isolates would be necessary to clarify this.

The various techniques differed in their ability to discriminate between individual isolates. Categorisation into four biotypes proved a very useful tool within the hospital for day-to-day assessment and clinical monitoring of the spread of MRSA, but supplementary methods such as immunoblotting and REFP analysis provided the "fine tuning" discrimination which is of value in the

detailed investigation of the origins and spread of infection with these organisms. The increased diversity revealed by these techniques is of benefit in these circumstances. The benefit of a quantitative approach to the assessment of strain relatedness, by means of Dice analysis, was considerable. Such methods reduce reliance on subjective evaluation of similarity with all its concomitant disadvantages.

All of the methods, with the exception of phage typing were able to type all the isolates. This is in agreement with the findings of Gaston and his co-workers (Gaston *et al*, 1988) who have previously found this to be the case for a variety of electrophoretic methods. Plasmid-free MRSA would naturally have been untypable by REFP analysis of plasmid DNA, but in such an event REFP analysis of chromosomal DNA could be used. (Hall and Jordens, 1988).

This study endorsed the findings by Cookson that the use of a combination of techniques yielded a breadth of epidemiological information which provided a clearer picture of the disposition of MRSA within the hospital (Cookson *et al*, 1986). The corroboration between the essentially disparate methods used in this study illustrates a number of interesting features of MRSA. First, they support the previous proposal that MRSA endemic in Glasgow Royal Infirmary are derived from two major clones. Second, the strain discrimination afforded by each method individually, indicates strain divergence in terms of both chromosomal and

extrachromosomal DNA. The parity between methods shows that neither strain nor plasmid shows independent instability. Data from both REFP analysis (extrachromosomal) and Western blots of exported proteins (predominantly chromosomal) strongly suggest that the two clones are diverging at different rates on the basis of inter-group differences and intra-group similarities. The predominant association of each of the two clones with different clinical units raises the intriguing possibility that the different divergence rates reflect differences in the selection pressures in the respective units, and may warrant further investigation.

The results obtained from the combination of methods described here suggests the potential for a strategic approach to the study of the epidemiology of MRSA. Simple biotyping could be employed to monitor large numbers of isolates. The increased resolution of the more detailed analytical techniques could then be focussed upon selected representative samples from therein. In this way maximum information could be derived from minimum expenditure of time, effort and money.

This work has attempted to examine MRSA in their proper epidemiological context. Particular emphasis has been placed on the analysis of plasmid DNA, and the results of these analyses have been related to information about a number of other properties of MRSA. The amount of epidemiological information yielded by combining the results of plasmid analysis with those from other techniques has provided a clearer picture of the nature of MRSA

within GRI. It seems certain that two major clones of MRSA are present within the hospital which demonstrate a number of differences in the various properties studied. What of the origins of these clones? The small plasmid data supports the view that these organisms evolved locally, although introduction from an exogenous source remains a possibility. But regardless of ultimate origin, either there was a common progenitor of the 2 major MRSA clones, or each arose from separate unique events to establish a separate lineage. MRSA isolates with properties which suggest they belonged to the aminoglycoside-sensitive group were first isolated during 1983 in GRI. Isolates of aminoglycoside-resistant MRSA were first encountered early in 1984. Thus if divergence from a common progenitor did occur, it must have been a fairly early event. Given the observed differences in a number of properties, it may well be that similar selection pressures at around the same time resulted in the establishment of two separate clones. It is unlikely that there will ever be an irrefutable answer because the original isolates are unfortunately unavailable for study to assess the degree of dissimilarity present at that time.

PATHOGENICITY FACTORS

ENTEROTOXIN PRODUCTION

The production of enterotoxin has been recognised increasingly as an important determinant of virulence in *Staph. aureus*. These biologically active substances, particularly

enterotoxins A and B, have been implicated in the pathogenesis of a number of clinical syndromes associated with staphylococcal infection. Their precise role in the majority of these syndromes remains to be elucidated. The main findings with regard to enterotoxin production in this study can be summarised as follows.

MSSA ISOLATES

If the pathogenicity of *Staph. aureus* is related to the production of enterotoxins it might be expected that isolates from "serious" infection episodes such as septicaemia, would be more likely to yield enterotoxins. Humphreys and his co-workers in Dublin have shown previously that more than 63 % of 52 blood culture isolates studied by them produced enterotoxins A, B, C or D either alone or in combination, whereas only 11 % of 27 nasal carriage isolates yielded enterotoxin, and in every case enterotoxin D alone (Humphreys *et al*, 1989). In this study 29/48 (60 %) of septicaemia (BC) isolates produced enterotoxins. This finding is in close agreement with their experience and the distribution of individual enterotoxins is similar (Table XIX). However, the findings for the nasal carriage isolates were strikingly different; 29/46 (63 %) of the Glasgow isolates produced at least one enterotoxin, although, as in Dublin, this was usually enterotoxin D.

Table XIX. Distribution of enterotoxin production in isolates from different sources: Dublin and Glasgow compared

Enterotoxin	Number(%) of isolates producing enterotoxin			
	Septicaemia		Nasal carriage	
	Dublin (n=52)	Glasgow (n=48)	Dublin (n=27)	Glasgow (n=46)
A	15 (29)	17 (35)	0 (0)	20 (43)
B	11 (21)	8 (17)	0 (0)	4 (9)
C	16 (31)	9 (19)	0 (0)	16 (35)
D	10 (19)	7 (15)	3 (11)	6 (13)

The phenotypic characteristics of the septicaemia isolates with regard to enterotoxin production were very similar in the isolates from both studies, and this may well reflect some enhanced propensity to produce invasive disease. The marked disparity in the ability of nasal carriage isolates to produce enterotoxin was intriguing. Firstly, it demonstrates that nasal carriage isolates from geographically distinct locations do not express this characteristic homogeneously, and underlines the need to establish an appropriate local representative collection of organisms when making comparisons. Secondly, it suggests that even if enterotoxin production is associated with enhanced pathogenicity, the ability of *Staph. aureus* to produce disease depends on other factors as well.

The proportion of enterotoxin producers amongst the RD *Staph. aureus* isolates (52 %) was slightly lower than amongst the BC and GP isolates (60 % and 63 % respectively), but not

significantly so. The distribution of the individual enterotoxins was again similar. However Jordens and co-workers did not detect enterotoxin production in any of ten clinical isolates of MSSA from the London Hospital (Jordens *et al*, 1989).

When the results from the BC isolates are considered it is not surprising that significant enterotoxin production is so common in other clinical isolates. Many of the septicaemic episodes were hospital acquired and consequently the organisms involved would form a subset of those found in other clinical lesions from patients within the hospital. It may be that the higher proportion of BC isolates that produced enterotoxins A, B, C and D when compared with other strains would have attained statistical significance if the sample size had been larger, and that enterotoxin production may be related to the invasive capabilities of these organisms. Conversely, it could be argued that other factors, unrelated to enterotoxin production are of most importance in determining the ability of an organism to produce septicaemia. None of the patients whose organisms made up the RD collection subsequently developed *Staph. aureus* septicaemia.

MRSA ISOLATES

A number of studies have suggested that enterotoxins may contribute to the pathogenicity of MRSA. Duckworth and Oppenheim (1986) described an association between the production of enterotoxins A and B and the pathogenesis of an illness

resembling toxic shock syndrome in two patients infected by EMRSA. All of 9 MRSA isolates from episodes of septicaemia in Dublin hospitals were found to produce enterotoxins A and B, either alone, or in combination (Humphreys *et al*, 1989). Jordens and co-workers have recently described the production of enterotoxin A alone in all of 6 EMRSA isolates studied (Jordens *et al*, 1989).

Enterotoxin production by aminoglycoside-sensitive MRSA in Glasgow Royal Infirmary reported in this study is very similar. Nineteen of twenty-three isolates (83 %) were enterotoxin A producers. Two of these isolates also produced enterotoxins B and C; a third elaborated enterotoxins B and C only. However, the data for the aminoglycoside-resistant MRSA were rather different. Nineteen of twenty-six isolates (73%) did not produce enterotoxins at all. Of the seven isolates which did produce enterotoxin, three isolates produced enterotoxin A alone, two isolates enterotoxins B and C, one isolate enterotoxins A, B and C, and one isolate enterotoxins A and C.

This difference between our results and those from other centres was interesting. It reinforces the idea, discussed elsewhere with regards to plasmid, biotype, immunoblot and phage type data, that there are two predominant clones of MRSA within the hospital; the enterotoxin A producing aminoglycoside-sensitive isolates and the predominantly non-enterotoxin producing aminoglycoside-resistant strains.

MRSA AND MSSA COMPARED

In the light of the marked differences in enterotoxin production between the aminoglycoside-sensitive and aminoglycoside-resistant MRSA, it was logical to consider each of these sub-groups separately when comparing the results with those of the MSSA. Enterotoxin production by the MSSA sub-groups was rather homogeneous, with the exception of the GP isolates which tended to have more enterotoxin C producers.

The significantly increased proportion of enterotoxin producers in the aminoglycoside sensitive MRSA was almost wholly due to the large number of strains that produced enterotoxin A. This increase in enterotoxin A producers, amongst MRSA isolates compared to MSSA, although less marked in the present work, is characteristic of the findings of previous studies (Humphreys *et al*, 1989; Jordens *et al*, 1989). However, the finding that enterotoxin production by aminoglycoside-resistant MRSA was less common than by MSSA was most intriguing. MRSA in GRI have been found to be efficient colonisers but have caused relatively few episodes of serious infection, which have usually been due to aminoglycoside-sensitive organisms (D R Baird, personal communication). Considering the small numbers of infections, the wide differences in the patients' underlying diseases, and the variety of other factors involved, it would be unwise to draw any firm conclusions from this association, but it is an area which merits further study in the future.

The most remarkable feature regarding enterotoxin production between the various collections was the difference in the number of strains elaborating enterotoxin A. Enterotoxin B production was remarkably homogeneous, regardless of the origin of the isolates. Hospital isolates of *Staph. aureus* were also consistent with regard to enterotoxin C although there was a small, but significant, increase in GP isolates producing this toxin. The complete absence of enterotoxin D producers amongst the MRSA isolates is interesting when the origin of these isolates is considered. Some of the results of this study have suggested that one or both MRSA clones may have been derived by local evolution from methicillin-sensitive strains. If this is so, it would have been expected to find some enterotoxin D producers amongst the MRSA. However the results do not preclude the existence of an enterotoxin D producing progenitor strain, which is either not present in this sample, or no longer demonstrates this phenotype due to loss of DNA or failure of expression.

HAEMOLYSINS

The *Staph. aureus* haemolysins, α-, β-, γ- and δ- are thought to exert their pathogenic effects by direct damage to cell membranes. They exhibit potent biological activity *in vitro*, and this is thought to reflect an important contribution by these toxins to the overall pathogenicity of isolates of *Staph. aureus*. The information obtained from the current study with regard to haemolysin production was considered as follows.

MSSA

In view of their cytotoxic effects, it might be expected that organisms isolated from septicaemic episodes would be more likely to elaborate haemolysins. Indeed, Humphreys (1989) has shown that in such isolates from Dublin hospitals, the frequencies of a–, γ– and δ– haemolysin production are in excess of 80%. In the current study, although slightly more β– haemolysin producers were found, the figures for the other haemolysins were considerably lower (Table XX). However, the figures for α– and γ– lysin were in closer agreement with those of Christenssen and Hedstrom (1986) based on a study of 88 septicaemia isolates of *Staph. aureus* which yielded figures of 58% and 24% respectively. Whilst the Dublin group found little difference in the frequency of haemolysin production in septicaemia and nasal carriage isolates, the current study identified substantially higher and lower numbers of γ– and δ– lysins respectively in the carriage group (Table XX).

With the exception of a very low frequency of γ– lysin production (28%), the distribution of haemolysin production in the RD group was similar to that of their hospital septicaemic counterparts, which again may reflect the fact that the BC isolates form a subset of the RD collection.

Table XX. Distribution of haemolysin production in isolates from different sources: Dublin and Glasgow compared

Haemolysin	Number(%) of isolates producing haemolysin			
	Septicaemia		Nasal carriage	
	Dublin (n=52)	Glasgow (n=48)	Dublin (n=27)	Glasgow (n=46)
α	43 (83)	28 (58)	25 (92)	27 (59)
β	3 (6)	9 (19)	3 (11)	6 (13)
γ	49 (94)	24 (50)	25 (93)	45 (98)
δ	49 (94)	25 (52)	25 (93)	11 (24)

MRSA

Both Jordens and Humphreys have previously shown that all, of five and nine isolates respectively, of MRSA which they tested produced α-, γ- and δ- haemolysins, whilst none elaborated β- lysin (Jordens *et al*, 1989; Humphreys *et al*, 1989). Whilst frequencies of haemolysin production, and particularly multiple haemolysin production, tended to be higher in MRSA from GRI than in other hospital isolates, the numbers of α- (73%), γ- (63%) and δ- (61%) lysin producers were not as high as in the Dublin and EMRSA studies. However, in contrast with these other studies, 28% of GRI MRSA isolates elaborated β- lysin.

Unlike the situation with regard to enterotoxin production, there were no marked differences in the frequencies of haemolysin

production in the aminoglycoside-sensitive and aminoglycoside-resistant MRSA groups.

MRSA AND MSSA COMPARED

The differences between the MSSA and MRSA with regard to haemolysin production reflected the tendency for the latter group to produce more haemolysins and to produce multiple haemolysins. α– haemolysin particularly was produced with higher frequency than in any of the MSSA collection. The slight increase in β– lysin producers amongst the MRSA did not reach significant levels, although there were clearly more δ– lysin producers amongst MRSA than amongst the RD MSSA. Whilst the similar frequencies of γ– lysin production in the MRSA and BC MSSA collections were significantly higher than in the RD MSSA, the very high frequency of γ– lysin producers amongst the community isolates was much greater than in any of these other groups.

GENERAL CONSIDERATIONS

Clearly the results of tests for pathogenicity factors in *Staph. aureus* demonstrated a number of similarities with previously published data, as well as some important differences. The major differences concern the RD MSSA and the MRSA data.

The GP MSSA data do not support the model of a simple relationship between enterotoxin and haemolysin production and pathogenicity. Enterotoxin production frequencies were very similar

to, and haemolysin production frequencies only marginally lower than, their counterparts from clinical infection episodes; enterotoxin C and γ– lysin frequencies were in fact significantly higher.

Amongst the MRSA, the aminoglycoside-sensitive isolates behaved in a very similar manner to their methicillin-resistant counterparts from elsewhere; high frequencies of enterotoxin A production were coupled with high frequencies of haemolysin production. However, while the aminoglycoside-resistant MRSA were indistinguishable with regard to haemolysin production, their very low frequency of enterotoxin production was quite different.

A thorough understanding of the mechanisms whereby microorganisms exert their pathogenic effects will play an increasing role in the development of strategies to combat microbial disease . Whilst "classical" exotoxin medicated diseases, such as tetanus or botulism are relatively straightforward to comprehend in terms of the extremely potent biological activity of individual toxins, these represent one end of a wide spectrum. At the other end of that spectrum organisms, hitherto regarded as innocuous and ill-equipped as pathogens, may result in overwhelming infections in the increasing numbers of compromised hosts in our hospitals. Between these extremes lie all shades of complexity of interactions between the host immune system and microbial pathogenic mechanisms.

There is little doubt that the enterotoxins and haemolysins of *Staph. aureus* are toxins of not inconsiderable potency. Equally in a

situation where all three MSSA groups produced these toxins with similar frequency there must be other factors which determined whether an individuals experience of the organism resulted in asymptomatic carriage or fatal septicaemia. These factors would have included other toxins and enzymes produced by the organisms, opportunity for deeper tissue invasion , humoral and cellular immune status, nutritional status, age and underlying disease of the patient. This multitude of variables confounds attempts to understand pathogenicity in terms of properties of the organism alone.

To some extent animal studies and in vitro systems can help us to standardise the relative pathogenic potential of organisms. In the long term it must be hoped that such studies in conjunction with an increased knowledge of immune mechanisms and quantitation of those factors which render our individual susceptible to infection will allow us to relate the properties of an organism to the disease produced in patients.

FUTURE DEVELOPMENTS

A continuation of the study to seek any further divergence and monitor the evolution of MRSA within GRI will be valuable. A longitudinal component was inherent in the design of this study, and it was clear that at the plasmid level there was some degree of stability. However, biotyping of more recent isolates has

demonstrated some increase in diversity (Dr D R Baird, personal communication).

The ecology of these strains is complex. This work has provided baseline data for the existing populations of MSSA and MRSA and, in addition, has established the analytical and interpretative framework for the further study of the MRSA. A strategic approach to the study of the evolution of these organisms within the hospital using a combination of conventional microbiological and molecular biological techniques is now possible.

Automation will facilitate the application of molecular methods to the analysis of epidemiological problems. Already commercial systems to perform rapid, automated immunoblotting are available, and have been used to provide answers to epidemiological questions (Stephenson *et al*, 1986). This technology will be used more widely to deal with the increasing problems of nosocomial infection and further developments in automation will inevitably encompass nucleic acid techniques, including plasmid profiles and REFPs. Automated and semi-automated molecular techniques are available now as diagnostic tests, and their extension into epidemiology is a natural progression. As automation decreases the demands on resource and expertise, molecular techniques in clinical microbiology will increase, and they will no longer be regarded as research methods. As has been shown in this study, it will be necessary to ensure adequate reference is

made to representative collections of organisms from appropriate "background" populations, rather than focussing on "outbreak" isolates alone, if valid comparisons are to be made.

One area worthy of particular attention is the development of more sophisticated analytical techniques. The availability of high performance densitometry systems and cheap, powerful microcomputers will permit the establishment and interrogation of large databases derived from protein and nucleic acid electrophoresis results. This will be an essential prerequisite for significant expansion in the use of these techniques, as it is time consuming and error-prone to make comparisons between isolates at present, particularly where either the numbers of strains and/or fragments involved is large or the strains do not appear on the same gel. To allow for variations in running conditions between one gel and another, particularly when comparing data from different centres, would require the incorporation of agreed standards in each gel. Indeed, to allow for the discontinuities within a single gel, it may even be necessary to include standards in many, if not every, track of the gel. In the latter case a further problem would be the need to differentiate the standards from the sample material. This internal standardisation would be required in addition to any agreed standardisation of protocols for restriction digestion schemes or preparation of antisera for immunoblotting, as have already been discussed.

Even if all these criteria are met, there is still a need to develop better methods for the quantitative analysis of the similarity of patterns generated by electrophoresis. The Dice co-efficient of similarity (Dice, 1945) has been used in this and other studies (Coia *et al*, 1990, Thomson-Carter and Pennington, 1989). Fourier transforms have been employed to quantitate similarity of immunoblots (Stephenson *et al*, 1986). However, it seems likely that improvements in these systems will be developed, utilising more sophisticated computer analysis. In addition, more specific features of the systems under study might be considered. For example, consider Dice analysis of the two hypothetical sets of REFPs of 2 different plasmids illustrated in Figure 20. In the comparison of the two plasmids in pair (a) the Dice co-efficient is:-

$$S_D = \frac{2 \times 4}{9} \times 100\% = 88.9\ \%$$

In the second pair (b), the Dice co-efficient is:-

$$S_D = \frac{2 \times 4}{9} \times 100\% = 88.9\ \%$$

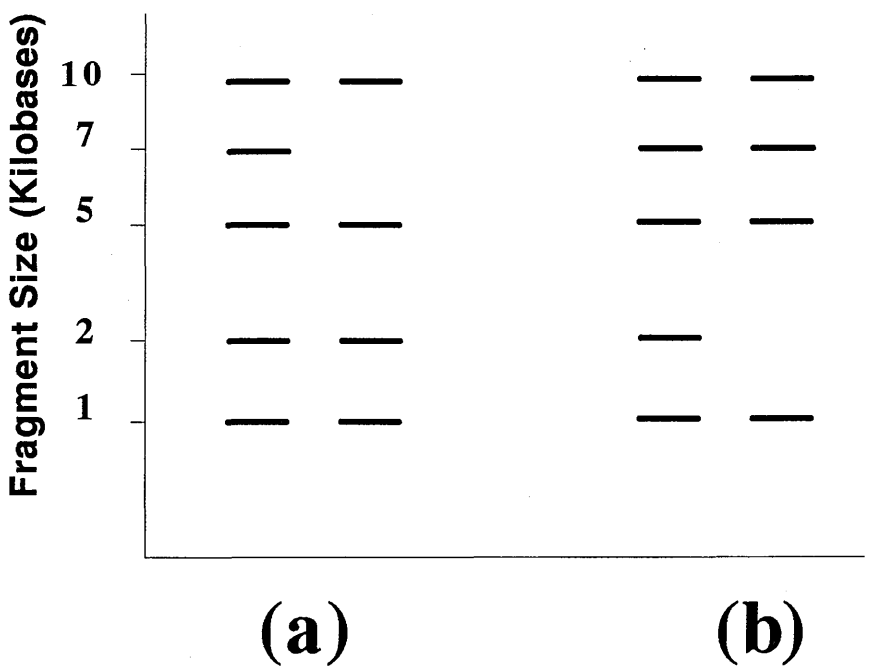


Figure 20. Restriction enzyme fragmentation patterns of two hypothetical related plasmids (a) and (b).

Since, the fragments in each lane are the digest products of a single plasmid, unlike the results of protein electrophoresis, where each band in a lane represents a discrete polypeptide, the molecular weight of the plasmid is equal to the sum of the fragment weights. It is therefore possible to deduce what proportion of the total plasmid molecular weight is contributed by each fragment, and to propose a modification of the Dice co-efficient whereby similarity (S) is:-

$$S = \frac{2 \times \text{mol wt of matching fragments}}{\text{mol wt of frag's in A + mol wt of frag's in B}} \times 100\%$$

When we apply this to the hypothetical REFPs the similarity of pair (a) now becomes:-

$$\text{Similarity} = \frac{36}{43} \times 100\ \% = 83.7\ \%$$

and for the second pair (b):-

$$\text{Similarity} = \frac{46}{48} \times 100\ \% = 95.8\ \%$$

This method takes more account of the relative contributions of larger and smaller fragments to the overall dissimilarity of the plasmids being compared. Clearly this system has limitations; it can only be used to compare single plasmid isolates, or single plasmids which have been segregated into plasmid free recipients. It is not particularly useful for chromosomal fingerprinting, because the large number of fragments and the difficulty of calculating accurately the total chromosomal size preclude calculation of the relative contributions of individual fragments. However, this is an example of how mathematical manipulations which take account of the underlying nature of the system may be employed in attempts to develop more discriminatory comparative indices.

It would be interesting to examine further the highly conserved small plasmids from the MRSA in GRI and to compare these more closely with other small plasmids found in *Staph. aureus*. The 2.7 kb *Hae*III restriction digest fragment is also intriguing. It would be interesting to clone this fragment and to use it to probe staphylococcal genomic DNA, and plasmids from other species, to establish how ubiquitous it is. One possibility is that it is involved in the encoding of some common function, another that it may be part of a regulatory or recognition sequence.

Chromosomal fingerprinting of *Staph. aureus* would be a logical extension of this study. Some preliminary work has been done using a modification of the method previously described for *Vibrio cholerae* (Mekalanos, 1983)(Figure 21).The results should be

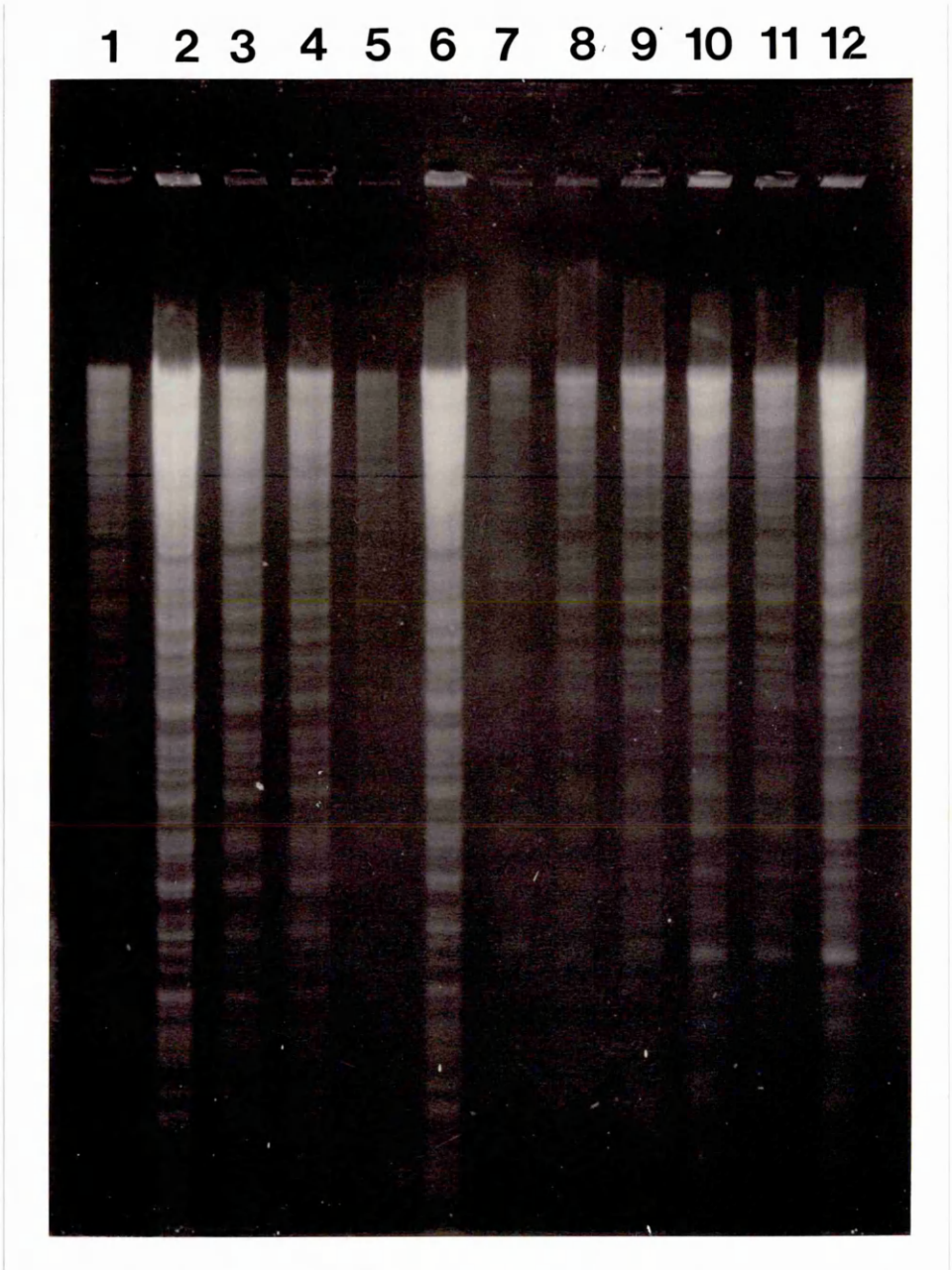


Figure 21. *Pst*I chromosomal fingerprints of *Staph. aureus*. Lanes 1-6 -strain 08886 (RD MSSA); lanes 7-12 -strain 18086 (MRSA(1)(R)).

particularly useful for the MSSA because more than half of the isolates were plasmid-free, and this precluded the use of plasmid REFPs as an epidemiological tool. Not surprisingly the number of fragments generated is very large, but more specific discrimination could be achieved, either by examining specific molecular size ranges, or by employing techniques such as field inversion gel electrophoresis to obtain better fragment resolution. In addition, Southern blotting using probes which recognise common repeated sequences, particularly ribosomal DNA genes, has been employed very successfully to examine the epidemiology of outbreaks of infection with a variety of organisms (Irino *et al*, 1988; Grimont *et al*, 1989).

Future investigation of pathogenicity determinants, could include the examination of various other toxins such as TSST-1, staphylokinase production and levels of coagulase production. However, the pathogenesis of staphylococcal disease is a very complex process, and development of our understanding of the factors involved is more likely to depend on elucidating the precise modes of action of the various components which contribute towards pathogenicity, than on extending the list of toxins and enzymes produced. This will require more sophisticated models of the pathogenic process in order to assess in greater detail the relative contributions of the various bacterial factors involved, and in addition aim to increase our understanding of the immune system and its interaction with the invading micro-organism.

CHAPTER 5

Conclusions

1) There were no differences in the prevalence, number or distribution of plasmids harboured by hospital and community isolates of MSSA.

2) There were significant differences in the prevalence, number and distribution of plasmids harboured by MSSA and MRSA. All MRSA harboured plasmids, whereas more than 50 % of MSSA were plasmid free.

3) Isolates with more than 1 plasmid were rare amongst plasmid-bearing MSSA, but represented the majority of MRSA; 85 % of MRSA harboured 2 plasmids.

4) The frequency distribution of plasmids in MSSA agreed well with that predicted by the modified Poisson model for plasmid distributions; the MRSA distribution did not fit the model and was consistent with the dissemination of a limited number of clones within the hospital.

5) Two size groups of plasmids, large and small, were detected. In MRSA the large plasmids comprised two main species and their molecular variants. The large MSSA plasmids were more diverse. The large plasmids found in MRSA and MSSA were different, but the small plasmids were identical irrespective of source.

6) There were no significant differences in the distribution and diversity of plasmids from early and late isolates of MRSA.

7) Dice analysis of MRSA REFPs identified two main subgroups, FP1 and FP2. These groups correlated with sensitivity to aminoglycoside antibiotics.

8) Phage typing, biotyping and immunoblot analysis defined subgroups of MRSA which correlated well with each other, and with those defined by REFP analysis.

9) Significantly more aminoglycoside-sensitive MRSA produced enterotoxin, predominantly enterotoxin A, than their MSSA counterparts. Conversely most aminoglycoside-resistant MRSA did not produce enterotoxins. These results concurred with the groupings defined by the various typing methods.

10) MRSA were more likely to produce multiple haemolysins than MSSA isolates. Aminoglycoside-sensitive and resistant MRSA were very similar with respect to haemolysin production, in contrast with enterotoxin production.

11) The results of antibiograms, biotyping, phage typing, immunoblotting, enterotoxin testing and REFPs were all compatible with the existence of 2 major clones of MRSA in GRI. The small plasmid data supported a hypothesis of local evolution of MRSA from endogenous MSSA.

APPENDIX 1

Origins, antibiotic sensitivities, number of plasmids harboured and REFPs[+] of *Staph. aureus* isolates

Isolate	Collection	Antibiotic															REFP	Plasmids
		Rd	Sm	Km	Cn	Va	W	Mup	P	Met	Ery	Da	Fd	Tet	C	Tec		
005986	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	19	1
006086	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
006186	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
006286	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	41	1
006386	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
006486	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
006586	BC	S	S	S	S	S	S	S	R	S	S	S	S	R	S	S	15	2
006686	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	42	1
006786	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
006886	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
014586	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0

[+]The REFP pattern number refers to accompanying Figure 22

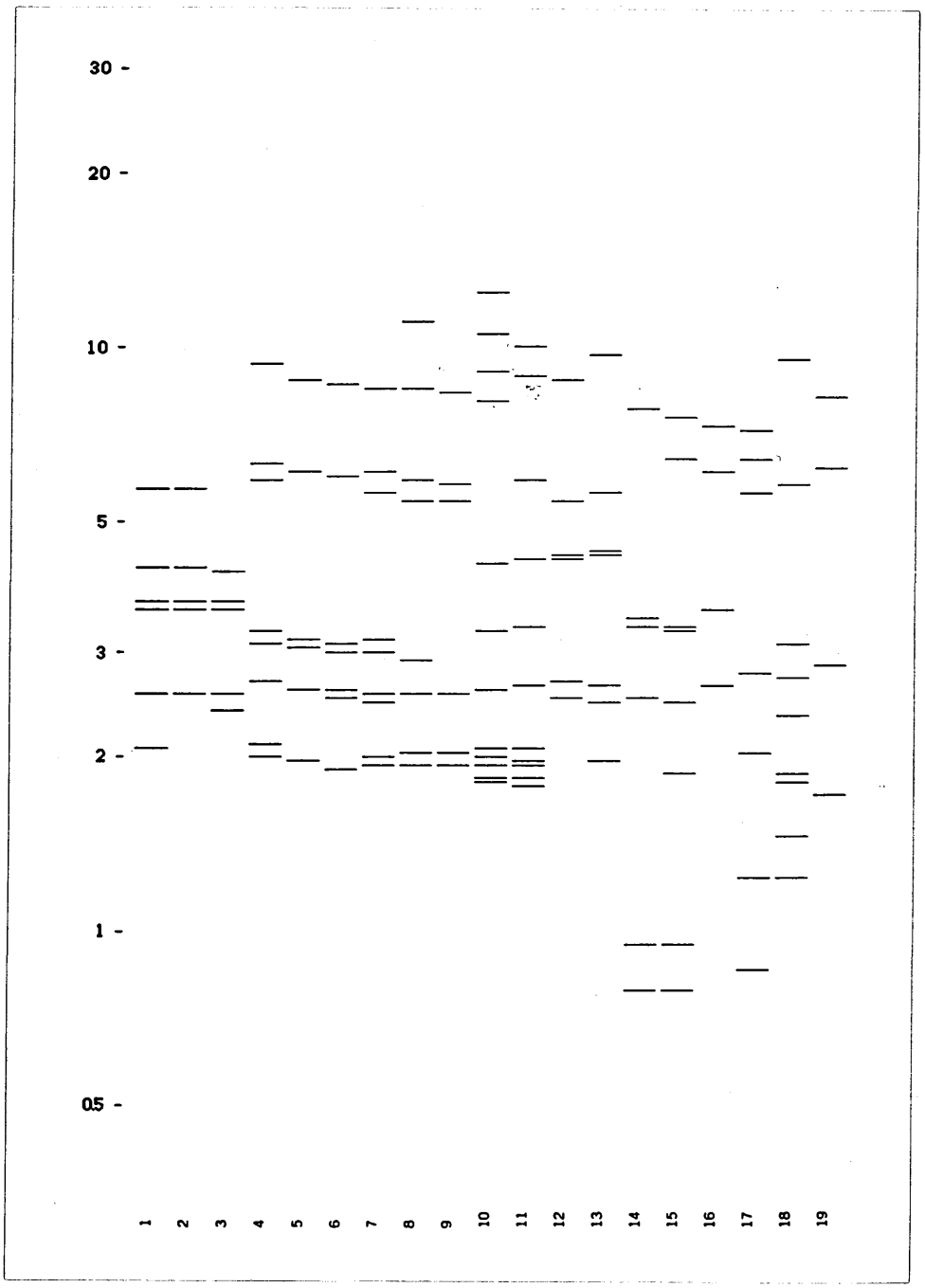


Figure 22a. *Hae*III restriction enzyme fragmentation patterns of *Staph. aureus*; patterns (1-19).

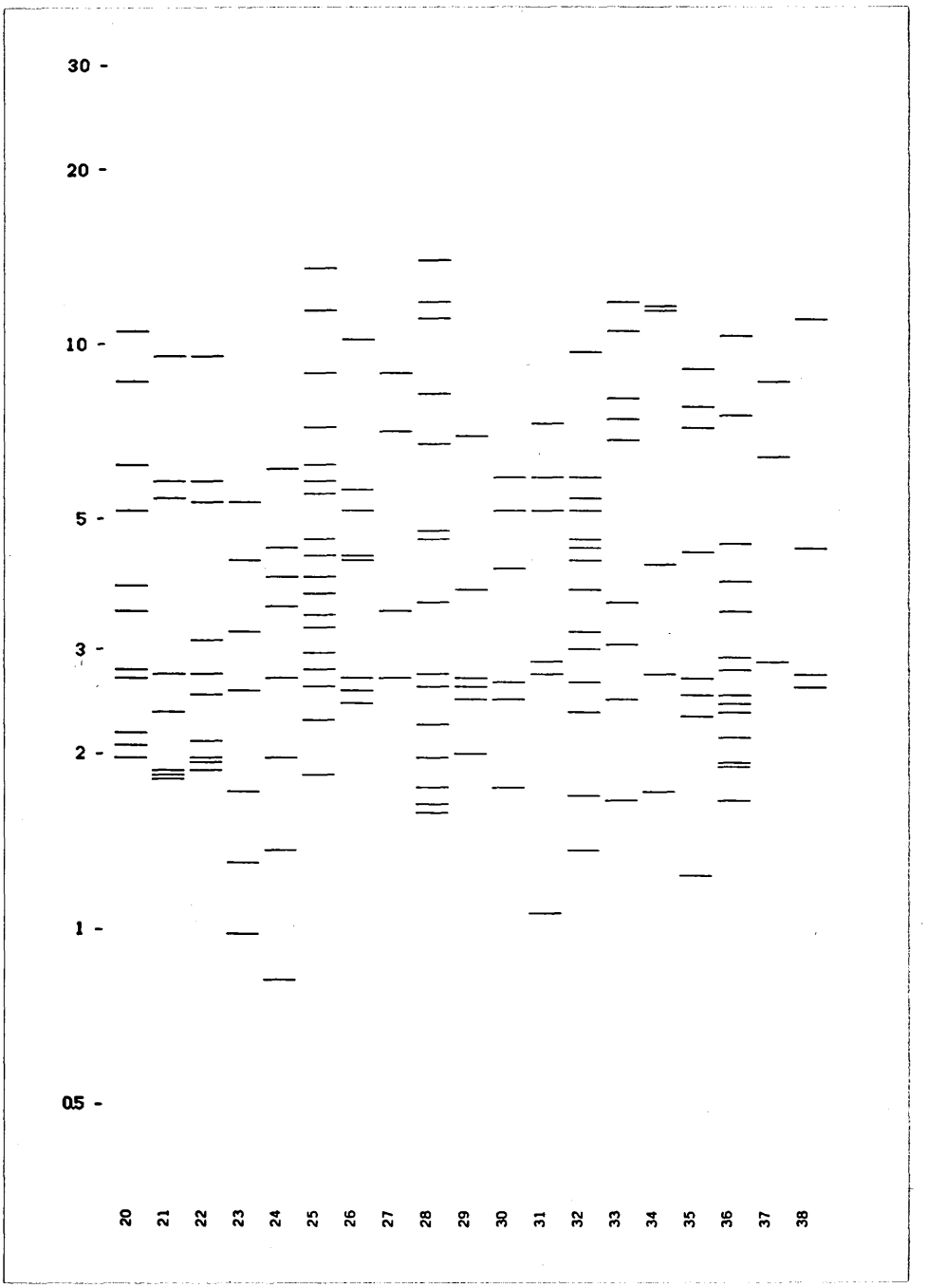


Figure 22b. *Hae*III restriction enzyme fragmentation patterns of *Staph. aureus*; patterns (20-38).

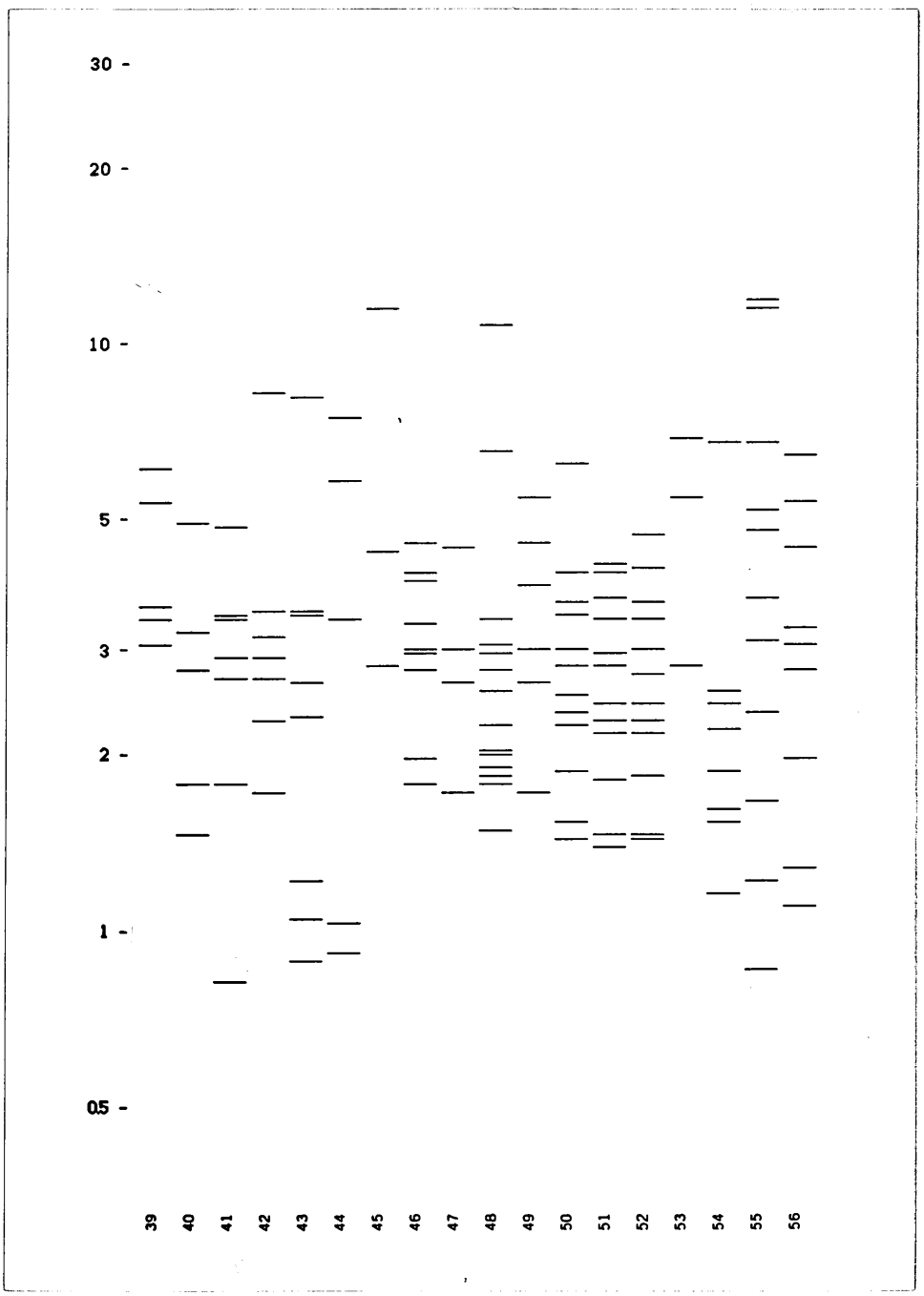


Figure 22c. *Hae*III restriction enzyme fragmentation patterns of *Staph. aureus*; patterns (39-56).

Origins, antibiotic sensitivities, number of plasmids harboured and REFPs[+] of *Staph. aureus* isolates

Isolate	Collection	Antibiotic															REFP	Plasmids
		Rd	Sm	Km	Cn	Va	W	Mup	P	Met	Ery	Da	Fd	Tet	C	Tec		
014686	BC	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	-	0
014786	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	19	1
014886	BC	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	-	0
015086	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	14	1
015286	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
015486	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
015586	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
015886	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	12	1
016086	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	43	1
016186	BC	S	S	S	S	S	S	S	R	S	S	S	R	S	S	S	-	0
016286	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0

[+]The REFP pattern number refers to accompanying Figure 22

Origins, antibiotic sensitivities, number of plasmids harboured and REFPs[+] of *Staph. aureus* isolates

Isolate	Collection	Antibiotic															REFP	Plasmids
		Rd	Sm	Km	Cn	Va	W	Mup	P	Met	Ery	Da	Fd	Tet	C	Tec		
016486	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
016586	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	14	1
016686	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
016786	BC	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	-	0
016886	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
016986	BC	S	R	R	S	S	S	S	R	S	R	S	R	R	S	S	-	0
017086	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	17	2
017186	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
017286	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
017386	BC	S	S	S	S	S	S	S	R	S	S	S	S	R	S	S	12	1
017486	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	52	1

[+]The REFP pattern number refers to accompanying Figure 22

Origins, antibiotic sensitivities, number of plasmids harboured and REFPs[+] of *Staph. aureus* isolates

Isolate	Collection	Antibiotic															REFP	Plasmids
		Rd	Sm	Km	Cn	Va	W	Mup	P	Met	Ery	Da	Fd	Tet	C	Tec		
017586	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
017686	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	12	1
017786	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	37	1
017886	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
017986	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	14	1
018186	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
018286	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	12	1
024286	BC	S	S	S	S	S	S	S	R	S	R	S	S	S	S	S	15	2
024386	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
024486	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
024586	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	38	1

[+]The REFP pattern number refers to accompanying Figure 22

Origins, antibiotic sensitivities, number of plasmids harboured and REFPs[+] of *Staph. aureus* isolates

Isolate	Collection	Antibiotic															REFP	Plasmids
		Rd	Sm	Km	Cn	Va	W	Mup	P	Met	Ery	Da	Fd	Tet	C	Tec		
024686	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
024786	BC	S	S	S	S	S	S	S	R	S	S	S	R	S	S	S	14	1
024886	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	14	1
024986	BC	S	S	S	S	S	S	S	R	S	R	S	R	R	S	S	50	1
025086	BC	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
025186	BC	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	-	0
020786	GP	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	44	1
020886	GP	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	-	0
020986	GP	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	45	1
021086	GP	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	12	1
021186	GP	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	12	1

[+]The REFP pattern number refers to accompanying Figure 22

Origins, antibiotic sensitivities, number of plasmids harboured and REFPs[+] of *Staph. aureus* isolates

Isolate	Collection	Antibiotic															REFP	Plasmids
		Rd	Sm	Km	Cn	Va	W	Mup	P	Met	Ery	Da	Fd	Tet	C	Tec		
021286	GP	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	-	0
021386	GP	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	46	1
021486	GP	S	S	S	S	S	S	S	R	S	S	S	S	R	S	S	16	1
021586	GP	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	47	1
021686	GP	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	48	1
021786	GP	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
021886	GP	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
021986	GP	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	-	0
022086	GP	S	S	S	S	S	S	S	R	S	S	S	S	R	S	S	15	1
022186	GP	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
022286	GP	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	49	1

[+]The REFP pattern number refers to accompanying Figure 22

Origins, antibiotic sensitivities, number of plasmids harboured and REFPs[+] of *Staph. aureus* isolates

Isolate	Collection	Antibiotic															REFP	Plasmids
		Rd	Sm	Km	Cn	Va	W	Mup	P	Met	Ery	Da	Fd	Tet	C	Tec		
022386	GP	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
022486	GP	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	16	1
022586	GP	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	-	0
022686	GP	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	16	1
022786	GP	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	-	0
022886	GP	S	S	S	S	S	S	S	R	S	S	S	S	R	S	S	15	2
022986	GP	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
023086	GP	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
023186	GP	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
023286	GP	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
023386	GP	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	50	1

[+]The REFP pattern number refers to accompanying Figure 22

Origins, antibiotic sensitivities, number of plasmids harboured and REFPs[+] of *Staph. aureus* isolates

Isolate	Collection	Antibiotic															REFP	Plasmids
		Rd	Sm	Km	Cn	Va	W	Mup	P	Met	Ery	Da	Fd	Tet	C	Tec		
023486	GP	S	S	S	S	S	S	S	R	S	S	S	S	R	S	S	12	1
023586	GP	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
023686	GP	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
023786	GP	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
023886	GP	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	-	0
023986	GP	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
024086	GP	S	S	S	S	S	S	S	R	S	S	S	S	R	S	S	56	2
024186	GP	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	51	1
025286	GP	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	-	0
025386	GP	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	-	0
025586	GP	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0

[+]The REFP pattern number refers to accompanying Figure 22

Origins, antibiotic sensitivities, number of plasmids harboured and REFPs[+] of *Staph. aureus* isolates

Isolate	Collection	Antibiotic															REFP	Plasmids
		Rd	Sm	Km	Cn	Va	W	Mup	P	Met	Ery	Da	Fd	Tet	C	Tec		
025686	GP	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	16	1
025786	GP	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	53	1
025886	GP	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	-	0
025986	GP	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	54	1
026086	GP	S	S	S	S	S	S	S	R	S	S	S	S	R	S	S	55	1
026186	GP	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
026286	GP	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	-	0
026386	GP	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
026486	GP	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	12	1
026586	GP	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	-	0
026686	GP	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	-	0

[+]The REFP pattern number refers to accompanying Figure 22

Origins, antibiotic sensitivities, number of plasmids harboured and REFPs[+] of *Staph. aureus* isolates

Isolate	Collection	Antibiotic															REFP	Plasmids
		Rd	Sm	Km	Cn	Va	W	Mup	P	Met	Ery	Da	Fd	Tet	C	Tec		
001286	RD	S	S	S	S	S	S	S	R	S	S	S	R	S	S	S	-	0
001386	RD	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
001486	RD	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	26	1
002186	RD	S	S	S	S	S	S	S	R	S	R	S	R	R	S	S	27	1
004086	RD	S	S	S	S	S	S	S	R	S	S	S	S	R	S	S	13	2
004986	RD	S	S	S	S	S	S	S	R	S	R	S	R	R	S	S	28	1
005086	RD	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	29	1
005386	RD	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	-	0
005586	RD	S	S	S	S	S	S	S	R	S	S	S	S	R	S	S	15	2
005686	RD	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
005886	RD	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0

[+]The REFP pattern number refers to accompanying Figure 22

Origins, antibiotic sensitivities, number of plasmids harboured and REFPs[+] of *Staph. aureus* isolates

Isolate	Collection	Antibiotic															REFP	Plasmids
		Rd	Sm	Km	Cn	Va	W	Mup	P	Met	Ery	Da	Fd	Tet	C	Tec		
008886	RD	S	S	S	S	S	S	S	R	S	S	S	S	R	S	S	-	0
008986	RD	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	30	1
009386	RD	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
009586	RD	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
009686	RD	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
009786	RD	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	16	1
009886	RD	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	31	1
009986	RD	S	S	S	S	S	S	S	R	S	S	S	S	R	S	S	15	2
010086	RD	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	14	1
010286	RD	S	S	S	S	S	S	S	R	S	R	S	S	R	S	S	-	0
010486	RD	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0

[+]The REFP pattern number refers to accompanying Figure 22

Origins, antibiotic sensitivities, number of plasmids harboured and REFPs[+] of *Staph. aureus* isolates

Isolate	Collection	Antibiotic															REFP	Plasmids
		Rd	Sm	Km	Cn	Va	W	Mup	P	Met	Ery	Da	Fd	Tet	C	Tec		
010586	RD	S	S	R	R	S	S	S	S	S	R	S	R	R	S	S	-	0
010686	RD	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	12	1
010886	RD	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	-	0
010986	RD	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	-	0
011186	RD	S	S	S	S	S	S	S	R	S	S	S	R	S	S	S	-	0
011386	RD	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
011486	RD	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
011586	RD	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
011886	RD	S	S	S	S	S	S	S	R	S	R	S	S	R	S	S	-	0
011986	RD	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	32	1
012386	RD	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0

[+]The REFP pattern number refers to accompanying Figure 22

Origins, antibiotic sensitivities, number of plasmids harboured and REFPs[+] of *Staph. aureus* isolates

Isolate	Collection	Antibiotic															REFP	Plasmids
		Rd	Sm	Km	Cn	Va	W	Mup	P	Met	Ery	Da	Fd	Tet	C	Tec		
012486	RD	S	S	S	S	S	S	S	R	S	S	S	S	R	S	S	-	0
013086	RD	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	-	0
013186	RD	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
013286	RD	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
013386	RD	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
013486	RD	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	-	0
013586	RD	S	S	S	S	S	S	S	R	S	S	S	S	R	S	S	-	0
013686	RD	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	-	0
013786	RD	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	14	1
013886	RD	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	-	0
013986	RD	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0

[+]The REFP pattern number refers to accompanying Figure 22

Origins, antibiotic sensitivities, number of plasmids harboured and REFPs[+] of *Staph. aureus* isolates

Isolate	Collection	Antibiotic															REFP	Plasmids
		Rd	Sm	Km	Cn	Va	W	Mup	P	Met	Ery	Da	Fd	Tet	C	Tec		
014086	RD	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
014286	RD	S	S	S	S	S	S	S	R	S	R	S	S	S	S	S	-	0
014386	RD	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
014486	RD	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	33	1
018586	RD	S	S	S	S	S	S	S	R	S	S	S	R	R	S	S	17	2
018686	RD	S	R	R	S	S	S	S	R	S	R	S	R	R	S	S	34	1
018786	RD	S	S	S	S	S	S	S	R	S	S	S	S	R	S	S	35	1
018886	RD	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	14	1
019086	RD	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
019186	RD	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0
019286	RD	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	-	0

[+]The REFP pattern number refers to accompanying Figure 22

Origins, antibiotic sensitivities, number of plasmids harboured and REFPs[+] of *Staph. aureus* isolates

Isolate	Collection	Antibiotic															REFP	Plasmids
		Rd	Sm	Km	Cn	Va	W	Mup	P	Met	Ery	Da	Fd	Tet	C	Tec		
019386	RD	S	S	S	S	S	S	S	R	S	S	S	S	R	S	S	17	2
019486	RD	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	36	1
019586	RD	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	37	1
019686	RD	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	38	1
030286	RD	S	S	S	S	S	S	S	R	S	S	S	S	R	S	S	-	0
030386	RD	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	39	1
030686	RD	S	S	S	S	S	S	S	R	S	R	S	S	S	S	S	40	1
001786	MRSA(1)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	4	2
001886	MRSA(1)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	4	2
002086	MRSA(1)(S)	S	S	S	S	S	R	S	R	R	S	S	S	S	S	S	20	2
002586	MRSA(1)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	5	1

[+]The REFP pattern number refers to accompanying Figure 22

Origins, antibiotic sensitivities, number of plasmids harboured and REFPs[+] of *Staph. aureus* isolates

Isolate	Collection	Antibiotic															REFP	Plasmids
		Rd	Sm	Km	Cn	Va	W	Mup	P	Met	Ery	Da	Fd	Tet	C	Tec		
005186	MRSA(1)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	4	2
006986	MRSA(1)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	5	1
008286	MRSA(1)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	4	2
008586	MRSA(1)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	5	1
008686	MRSA(1)(S)	S	R	S	S	S	R	S	R	R	S	S	S	S	S	S	9	1
008786	MRSA(1)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	5	1
015786	MRSA(1)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	4	2
015986	MRSA(1)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	4	2
027086	MRSA(1)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	4	2
027286	MRSA(1)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	4	2
027486	MRSA(1)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	5	1

[+]The REFP pattern number refers to accompanying Figure 22

Origins, antibiotic sensitivities, number of plasmids harboured and REFPs[+] of *Staph. aureus* isolates

Isolate	Collection	Antibiotic															REFP	Plasmids
		Rd	Sm	Km	Cn	Va	W	Mup	P	Met	Ery	Da	Fd	Tet	C	Tec		
027686	MRSA(1)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	4	2
027886	MRSA(1)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	5	1
027986	MRSA(1)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	18	3
028086	MRSA(1)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	18	3
028586	MRSA(1)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	4	2
028886	MRSA(1)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	4	2
029086	MRSA(1)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	5	1
029186	MRSA(1)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	22	3
029486	MRSA(1)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	21	2
029786	MRSA(1)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	4	2
030586	MRSA(1)(S)	S	S	S	S	S	S	R	R	R	R	S	S	R	S	S	25	4

[+]The REFP pattern number refers to accompanying Figure 22

Origins, antibiotic sensitivities, number of plasmids harboured and REFPs[+] of *Staph. aureus* isolates

Isolate	Collection	Antibiotic															REFP	Plasmids
		Rd	Sm	Km	Cn	Va	W	Mup	P	Met	Ery	Da	Fd	Tet	C	Tec		
002686	MRSA(1)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
005286	MRSA(1)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	24	2
008086	MRSA(1)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
008186	MRSA(1)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
008386	MRSA(1)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
008486	MRSA(1)(R)	S	R	R	R	S	R	S	R	R	R	S	R	R	R	S	1	2
014986	MRSA(1)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
015386	MRSA(1)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
015686	MRSA(1)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
018086	MRSA(1)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
027186	MRSA(1)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2

[+]The REFP pattern number refers to accompanying Figure 22

Origins, antibiotic sensitivities, number of plasmids harboured and REFPs[+] of *Staph. aureus* isolates

Isolate	Collection	Antibiotic															REFP	Plasmids
		Rd	Sm	Km	Cn	Va	W	Mup	P	Met	Ery	Da	Fd	Tet	C	Tec		
027386	MRSA(1)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
027586	MRSA(1)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
027786	MRSA(1)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
028186	MRSA(1)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
028386	MRSA(1)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
028486	MRSA(1)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
028686	MRSA(1)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
028786	MRSA(1)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
028986	MRSA(1)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
029286	MRSA(1)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
029386	MRSA(1)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2

[+]The REFP pattern number refers to accompanying Figure 22

Origins, antibiotic sensitivities, number of plasmids harboured and REFPs[+] of *Staph. aureus* isolates

Isolate	Collection	Antibiotic															REFP	Plasmids
		Rd	Sm	Km	Cn	Va	W	Mup	P	Met	Ery	Da	Fd	Tet	C	Tec		
029586	MRSA(1)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
029686	MRSA(1)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
029886	MRSA(1)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
030486	MRSA(1)(R)	S	R	R	R	S	S	S	R	R	R	S	R	S	S	S	2	1
009887	MRSA(2)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	6	1
009987	MRSA(2)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	7	2
010087	MRSA(2)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	8	2
010187	MRSA(2)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	9	2
010287	MRSA(2)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	5	1
010387	MRSA(2)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	7	2
010487	MRSA(2)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	4	2

[+]The REFP pattern number refers to accompanying Figure 22

Origins, antibiotic sensitivities, number of plasmids harboured and REFPs[+] of *Staph. aureus* isolates

Isolate	Collection	Antibiotic															REFP	Plasmids
		Rd	Sm	Km	Cn	Va	W	Mup	P	Met	Ery	Da	Fd	Tet	C	Tec		
010587	MRSA(2)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	4	2
010687	MRSA(2)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	10	2
010787	MRSA(2)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	11	3
010887	MRSA(2)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	4	2
010987	MRSA(2)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	4	2
011087	MRSA(2)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	9	2
011187	MRSA(2)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	4	2
011287	MRSA(2)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	5	1
011387	MRSA(2)(S)	S	S	S	S	S	S	R	R	R	R	S	S	R	S	S	4	2
011487	MRSA(2)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	4	2
011587	MRSA(2)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	5	1

[+]The REFP pattern number refers to accompanying Figure 22

Origins, antibiotic sensitivities, number of plasmids harboured and REFPs[+] of *Staph. aureus* isolates

Isolate	Collection	Antibiotic															REFP	Plasmids
		Rd	Sm	Km	Cn	Va	W	Mup	P	Met	Ery	Da	Fd	Tet	C	Tec		
011687	MRSA(2)(S)	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S	4	2
011787	MRSA(2)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
011887	MRSA(2)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
011987	MRSA(2)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
012087	MRSA(2)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
012187	MRSA(2)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
012287	MRSA(2)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
012387	MRSA(2)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
012487	MRSA(2)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
012587	MRSA(2)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
012687	MRSA(2)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2

[+]The REFP pattern number refers to accompanying Figure 22

Origins, antibiotic sensitivities, number of plasmids harboured and REFPs[+] of *Staph. aureus* isolates

Isolate	Collection	Antibiotic															REFP	Plasmids
		Rd	Sm	Km	Cn	Va	W	Mup	P	Met	Ery	Da	Fd	Tet	C	Tec		
012787	MRSA(2)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
012887	MRSA(2)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
012987	MRSA(2)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
013187	MRSA(2)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	3	1
013287	MRSA(2)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	7	2
013487	MRSA(2)(R)	S	S	R	R	S	S	S	R	R	R	S	S	R	S	S	7	2
013587	MRSA(2)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	R	S	2	1
013687	MRSA(2)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
013787	MRSA(2)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
013887	MRSA(2)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
013987	MRSA(2)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2

[+]The REFP pattern number refers to accompanying Figure 22

Origins, antibiotic sensitivities, number of plasmids harboured and REFPs[+] of *Staph. aureus* isolates

Isolate	Collection	Antibiotic															REFP	Plasmids
		Rd	Sm	Km	Cn	Va	W	Mup	P	Met	Ery	Da	Fd	Tet	C	Tec		
014087	MRSA(2)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
014187	MRSA(2)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
014287	MRSA(2)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
014387	MRSA(2)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
014487	MRSA(2)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
014587	MRSA(2)(R)	S	R	R	R	S	S	S	R	R	R	S	S	S	S	S	2	1
014687	MRSA(2)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2
014787	MRSA(2)(R)	S	R	R	R	S	S	S	R	R	R	S	S	R	S	S	1	2

[+]The REFP pattern number refers to accompanying Figure 22

APPENDIX 2

Phage typing of methicillin-resistant *Staph. aureus*

Isolate	Standard set (RTD)	Additional reactions (RTD x 100)	Extended set	Supplementary for MRSA
1886	6/47/53/54/75/83A/85	Nil	NA	616/617/622/623/626
2586	85	53	NA	616/617/622/625/630
2686	75/84	52	83C/932	NA
5186	6/47/53/54/75/83A	29/52/52A/79/80/42E/85/81	88A/90/83C/932	616/617/622/623/626
5286	NT	75	NA	617/622/630
6986	53/75/83A/85	Nil	NA	617/623
8086	NT	75/84	NA	NT
8186	NT	75/84	NA	NT
8286	NT	75/84	NA	NT
8386	NA	NA	NA	NA
8686	NA	NA	NA	NA
8786	53/85	29/52/52A/79/80/95/54/96	88A/90/932	616/617/622/626
14986	NT	75/84	NA	NT
15386	NT	75/84	NA	NT
15686	NT	75	NT	NT
15786	53/85	53/85	NA	616/617/622/626
15986	53/83A/85	29/52/52A/79/80/54/96	88A/90/83C/932	616/617/622/626
18086	NA	NA	NA	NA
27086	53/85	29/79/80	88A/90/932	616/617/622/626
27186	NT	75/84	NT	NT
27286	53/83A/85	54	90	NT
27386	NT	75	NT	NT
27486	53/85	29/52/52A/79/80/96	88A/90/932	616/617/622/626
27586	NT	75/84	NT	NT
27686	53/75/83A/85	29/52/52A/79/80/95/6/96	88A/90/83C/932	616/617/622/626
27786	NT	75	NT	NT
27886	53/85	29/52/79/80/95/96	88A/90/932	616/617/622/626/630
27986	53/85	29/52/52A/79/80/95	88A/90/932	617/622/626
28086	53/75/83A/85	6/47/54	88A/90/83C	617
28386	NT	75/84	NT	NT
28586	NT	Nil	90/932	617/622
28686	NT	47/75/77/83A/84/85	NT	620/630

NA = Not available; NT = non-typable; Nil = No additional reactions at 100 x RTD

Phage typing of methicillin-resistant *Staph. aureus*

Isolate	Standard set (RTD)	Additional reactions (RTD x 100)	Extended set	Supplementary for MRSA
28786	NT	75	NT	NT
28886	53/85	29	88A/90/932	616/617/622/626/630
28986	NT	75/84	NT	618/622
29086	53/83A/85	29/52/79/80/95/54/96	88A/90/83C/932	616/617/622/626/630
29186	6/53/54/75/83A/85	42E/47/81	88A/90/83C/932	617/622/630
29286	NT	75	NT	NT
29386	NT	75/84	NT	NT
29486	6/53/75/83A/85	6/47/54	88A/90/83C/932	617/623
29586	NT	75	NT	NT
29686	NT	53/75/84	NT	NT
29886	NT	75	NT	NT
30486	77/84	Nil	NA	617/618/622
30586	29/52/52A/79/80/95/53/75/77/85	77/83A	88A/90/83C/932	616/617/622/626/629/630

NA = Not available; NT = non-typable; Nil = No additional reactions at 100 x RTD

APPENDIX 3

Enterotoxin and Haemolysin production by isolates from different sources

Isolate	Collection	Enterotoxins				Haemolysins			
		A	B	C	D	α	β	γ	δ
005986	BC	No	Yes	Yes	No	No	No	Yes	Yes
006086	BC	No	No	No	No	No	Yes	No	No
006186	BC	No	No	No	No	No	No	Yes	No
006286	BC	No	No	No	No	Yes	No	No	Yes
006386	BC	Yes	No	No	No	Yes	Yes	No	Yes
006486	BC	Yes	No	No	No	No	No	Yes	Yes
006586	BC	Yes	No	No	No	Yes	No	Yes	Yes
006686	BC	No	No	No	No	No	No	No	No
006786	BC	No	No	No	No	Yes	No	No	No
006886	BC	No	No	No	No	Yes	No	No	Yes
014586	BC	No	No	No	No	Yes	Yes	No	No
014686	BC	No	No	No	No	Yes	No	No	Yes

Enterotoxin and Haemolysin production by isolates from different sources

Isolate	Collection	Enterotoxins				Haemolysins			
		A	B	C	D	α	β	γ	δ
014786	BC	Yes	Yes	No	No	No	No	Yes	No
014886	BC	Yes	No	No	No	Yes	No	No	No
015286	BC	No	No	No	No	Yes	Yes	No	Yes
015486	BC	No	Yes	No	No	No	No	Yes	No
015586	BC	No	No	Yes	No	Yes	Yes	No	Yes
015886	BC	No	No	Yes	Yes	No	No	Yes	No
016086	BC	Yes	Yes	Yes	Yes	No	No	No	Yes
016186	BC	No	No	No	No	Yes	No	Yes	No
016286	BC	No	No	No	No	Yes	Yes	Yes	Yes
016486	BC	Yes	No	No	No	Yes	No	No	No
016586	BC	No	No	No	No	Yes	No	No	Yes
016686	BC	No	No	No	No	Yes	No	No	Yes

Enterotoxin and Haemolysin production by isolates from different sources

Isolate	Collection	Enterotoxins				Haemolysins			
		A	B	C	D	α	β	γ	δ
016786	BC	No	No	Yes	No	Yes	No	No	Yes
016886	BC	Yes	Yes	Yes	No	Yes	Yes	No	Yes
016986	BC	No	No	No	No	Yes	Yes	No	Yes
017086	BC	No	Yes	No	No	Yes	No	No	Yes
017186	BC	No	No	No	Yes	No	No	Yes	No
017286	BC	Yes	No	No	No	No	No	Yes	No
017386	BC	No	No	No	Yes	No	No	Yes	No
017486	BC	No	No	No	Yes	No	No	Yes	No
017586	BC	No	No	No	No	Yes	No	No	Yes
017786	BC	No	Yes	No	No	Yes	No	No	Yes
017886	BC	Yes	No	No	No	No	No	Yes	Yes
017986	BC	No	No	No	No	Yes	No	No	No

Enterotoxin and Haemolysin production by isolates from different sources

Isolate	Collection	Enterotoxins				Haemolysins			
		A	B	C	D	α	β	γ	δ
018186	BC	Yes	No	No	No	Yes	No	No	Yes
018286	BC	No	No	Yes	Yes	Yes	No	No	Yes
024286	BC	Yes	No	No	No	No	No	Yes	No
024386	BC	Yes	No	No	No	No	No	Yes	No
024486	BC	Yes	No	Yes	Yes	No	No	Yes	No
024586	BC	No	No	No	No	Yes	No	Yes	No
024686	BC	No	No	No	No	No	No	Yes	No
024786	BC	Yes	No	No	No	No	No	Yes	No
024886	BC	No	No	No	No	Yes	No	Yes	Yes
024986	BC	Yes	No	No	No	Yes	No	Yes	Yes
025086	BC	Yes	No	No	No	No	No	Yes	No
025186	BC	No	Yes	Yes	No	Yes	Yes	Yes	Yes

Enterotoxin and Haemolysin production by isolates from different sources

Isolate	Collection	Enterotoxins				Haemolysins			
		A	B	C	D	α	β	γ	δ
020786	GP	No	No	No	No	Yes	No	Yes	No
020886	GP	Yes	No	No	No	Yes	No	Yes	No
020986	GP	Yes	No	Yes	No	Yes	No	Yes	Yes
021086	GP	Yes	No	Yes	Yes	Yes	No	Yes	No
021186	GP	Yes	No	Yes	Yes	No	No	Yes	No
021286	GP	Yes	No	Yes	No	No	No	Yes	No
021386	GP	No	No	No	No	No	No	Yes	No
021486	GP	Yes	No	No	No	Yes	No	Yes	Yes
021586	GP	No	No	No	No	Yes	No	Yes	No
021686	GP	No	No	Yes	No	No	No	Yes	No
021786	GP	Yes	No	No	No	No	No	Yes	No
021986	GP	No	No	Yes	No	Yes	No	Yes	No

Enterotoxin and Haemolysin production by isolates from different sources

Isolate	Collection	Enterotoxins				Haemolysins			
		A	B	C	D	α	β	γ	δ
022186	GP	Yes	No	No	No	No	No	Yes	No
022286	GP	No	No	No	No	No	No	Yes	No
022386	GP	Yes	No	Yes	No	No	Yes	Yes	No
022486	GP	No	No	No	No	Yes	No	Yes	No
022586	GP	No	No	Yes	No	Yes	No	Yes	No
022686	GP	No	No	No	No	No	No	No	No
022786	GP	No	No	Yes	Yes	No	Yes	Yes	Yes
022886	GP	No	No	No	No	Yes	No	Yes	Yes
022986	GP	Yes	No	No	No	No	No	Yes	No
023086	GP	Yes	No	No	No	Yes	No	Yes	No
023186	GP	Yes	Yes	Yes	No	No	Yes	Yes	Yes
023286	GP	Yes	No	No	Yes	No	No	Yes	No

Enterotoxin and Haemolysin production by isolates from different sources

Isolate	Collection	Enterotoxins				Haemolysins			
		A	B	C	D	α	β	γ	δ
023386	GP	No	No	No	No	Yes	No	Yes	No
023486	GP	Yes	No	Yes	Yes	Yes	No	Yes	No
023586	GP	Yes	No	No	No	No	No	Yes	No
023686	GP	Yes	No	No	No	No	No	Yes	No
023786	GP	No	Yes	No	No	No	No	Yes	No
023886	GP	No	No	No	No	Yes	No	Yes	No
023986	GP	No	Yes	Yes	No	Yes	No	Yes	Yes
024086	GP	No	Yes	Yes	No	Yes	No	Yes	No
024186	GP	No	No	No	No	Yes	No	Yes	Yes
025586	GP	Yes	No	No	No	No	No	Yes	Yes
025686	GP	No	No	No	No	Yes	No	Yes	No
025786	GP	No	No	No	No	Yes	No	Yes	No

Enterotoxin and Haemolysin production by isolates from different sources

Isolate	Collection	Enterotoxins				Haemolysins			
		A	B	C	D	α	β	γ	δ
025886	GP	Yes	No	No	No	No	No	Yes	No
025986	GP	No	No	No	No	Yes	Yes	Yes	No
026086	GP	No	No	No	No	Yes	No	Yes	No
026186	GP	No	No	No	No	Yes	No	Yes	No
026286	GP	Yes	No	Yes	No	Yes	No	Yes	No
026386	GP	No	No	No	No	Yes	Yes	Yes	Yes
026486	GP	No	No	Yes	No	Yes	No	Yes	No
026586	GP	No	No	Yes	Yes	Yes	No	Yes	No
026686	GP	No	No	No	No	Yes	No	Yes	Yes
029486	GP	Yes	No	No	No	No	Yes	Yes	Yes
001286	RD	Yes	No	No	No	No	No	Yes	No
001386	RD	No	No	Yes	No	No	Yes	No	Yes

Enterotoxin and Haemolysin production by isolates from different sources

Isolate	Collection	Enterotoxins				Haemolysins			
		A	B	C	D	α	β	γ	δ
001486	RD	No	No	Yes	Yes	No	No	No	Yes
002186	RD	No	No	No	No	Yes	Yes	No	Yes
004086	RD	No	No	No	Yes	No	Yes	No	Yes
004986	RD	Yes	No	No	No	Yes	Yes	No	Yes
005086	RD	Yes	No	No	No	Yes	Yes	No	Yes
005586	RD	Yes	No	No	No	No	No	Yes	Yes
005686	RD	No	No	Yes	No	No	No	Yes	Yes
005886	RD	No	No	No	No	No	No	No	Yes
008886	RD	Yes	No	No	No	No	No	Yes	No
008986	RD	No	Yes	No	No	Yes	No	No	Yes
009386	RD	Yes	Yes	No	No	No	Yes	No	No
009586	RD	Yes	No	No	No	No	No	Yes	No

Enterotoxin and Haemolysin production by isolates from different sources

Isolate	Collection	Enterotoxins				Haemolysins			
		A	B	C	D	α	β	γ	δ
009686	RD	No	No	No	No	No	No	Yes	No
009786	RD	No	No	No	No	No	No	Yes	Yes
009886	RD	No	No	Yes	No	Yes	No	No	No
009986	RD	Yes	No	No	No	No	No	Yes	Yes
010086	RD	No	No	No	No	Yes	No	No	Yes
010286	RD	No	No	No	No	Yes	No	No	Yes
010486	RD	Yes	No	No	No	No	No	Yes	No
010586	RD	Yes	No	No	No	Yes	No	No	Yes
010686	RD	No	No	Yes	Yes	Yes	No	No	Yes
010886	RD	No	No	No	No	Yes	No	No	No
010986	RD	No	No	No	No	No	No	No	Yes
011186	RD	No	Yes	No	No	Yes	Yes	No	No

Enterotoxin and Haemolysin production by isolates from different sources

Isolate	Collection	Enterotoxins				Haemolysins			
		A	B	C	D	α	β	γ	δ
011386	RD	No	Yes	No	No	Yes	No	No	Yes
011486	RD	No	No	No	No	Yes	No	No	Yes
011586	RD	Yes	No	No	No	Yes	Yes	No	No
011886	RD	No	No	No	No	Yes	No	No	Yes
011986	RD	No	No	No	No	No	Yes	No	No
012386	RD	No	Yes	No	No	Yes	No	No	Yes
012486	RD	No	Yes	No	No	No	No	No	No
013086	RD	No	No	No	No	Yes	No	No	Yes
013186	RD	No	No	No	No	No	No	Yes	No
013286	RD	Yes	No	No	No	No	Yes	Yes	No
013386	RD	Yes	No	No	No	No	No	No	No
013486	RD	No	No	No	No	Yes	No	No	No

Enterotoxin and Haemolysin production by isolates from different sources

Isolate	Collection	Enterotoxins				Haemolysins			
		A	B	C	D	α	β	γ	δ
013586	RD	Yes	No	No	No	No	No	No	No
013686	RD	Yes	No	No	No	Yes	No	No	No
013786	RD	No	No	No	No	No	No	Yes	Yes
013886	RD	No	No	No	No	No	Yes	Yes	Yes
013986	RD	No	No	No	No	No	No	No	No
014086	RD	No	No	No	No	Yes	No	No	No
014286	RD	Yes	No	No	No	Yes	No	No	Yes
014386	RD	No	No	No	No	Yes	No	No	Yes
014486	RD	No	No	No	No	No	No	No	Yes
018586	RD	No	No	No	No	Yes	No	No	Yes
018686	RD	No	No	No	No	Yes	No	No	Yes
018786	RD	Yes	Yes	No	Yes	No	No	Yes	No

Enterotoxin and Haemolysin production by isolates from different sources

Isolate	Collection	Enterotoxins				Haemolysins			
		A	B	C	D	α	β	γ	δ
019086	RD	No	Yes	No	No	Yes	Yes	No	Yes
019186	RD	No	No	No	No	No	No	No	No
019286	RD	No	No	No	No	Yes	Yes	No	Yes
019386	RD	No	No	No	No	Yes	No	No	Yes
019486	RD	No	No	No	No	Yes	No	Yes	Yes
019586	RD	No	No	No	No	Yes	Yes	No	No
019686	RD	Yes	No	No	No	Yes	Yes	No	Yes
030386	RD	No	No	No	No	Yes	No	Yes	Yes
001786	MRSA(S)	Yes	No	No	No	Yes	Yes	No	Yes
001886	MRSA(S)	Yes	No	No	No	Yes	Yes	No	Yes
002586	MRSA(S)	Yes	No	No	No	Yes	Yes	No	Yes
005186	MRSA(S)	Yes	No	No	No	Yes	Yes	Yes	Yes

Enterotoxin and Haemolysin production by isolates from different sources

Isolate	Collection	Enterotoxins				Haemolysins			
		A	B	C	D	α	β	γ	δ
006986	MRSA(S)	Yes	No	No	No	Yes	No	No	Yes
008286	MRSA(S)	Yes	Yes	Yes	No	No	Yes	No	No
008786	MRSA(S)	Yes	No	No	No	Yes	No	No	Yes
015986	MRSA(S)	Yes	No	No	No	Yes	No	No	Yes
027086	MRSA(S)	Yes	No	No	No	Yes	No	Yes	Yes
027286	MRSA(S)	Yes	No	No	No	Yes	No	Yes	Yes
027486	MRSA(S)	Yes	No	No	No	Yes	No	Yes	Yes
027686	MRSA(S)	Yes	No	No	No	Yes	No	Yes	No
027886	MRSA(S)	No	No	No	No	Yes	Yes	Yes	Yes
027986	MRSA(S)	Yes	No	No	No	Yes	No	Yes	No
028086	MRSA(S)	Yes	No	No	No	Yes	No	Yes	No
028586	MRSA(S)	Yes	No	No	No	Yes	No	Yes	Yes

Enterotoxin and Haemolysin production by isolates from different sources

Isolate	Collection	Enterotoxins				Haemolysins			
		A	B	C	D	α	β	γ	δ
028886	MRSA(S)	No	No	No	No	No	Yes	Yes	No
029086	MRSA(S)	Yes	No	No	No	No	Yes	Yes	No
029186	MRSA(S)	Yes	No	No	No	No	No	Yes	Yes
029786	MRSA(S)	Yes	No	No	No	No	No	Yes	Yes
008586	MRSA(S)	No	Yes	Yes	No	No	No	No	No
008686	MRSA(S)	Yes	Yes	Yes	No	No	No	No	No
030586	MRSA(S)	No	No	No	No	No	No	No	No
002686	MRSA(R)	Yes	No	No	No	Yes	Yes	Yes	Yes
005286	MRSA(R)	No	No	No	No	Yes	Yes	No	Yes
008086	MRSA(R)	No	No	No	No	Yes	Yes	No	Yes
008186	MRSA(R)	No	No	No	No	No	No	No	No
008386	MRSA(R)	No	No	No	No	Yes	Yes	No	Yes

Enterotoxin and Haemolysin production by isolates from different sources

Isolate	Collection	Enterotoxins				Haemolysins			
		A	B	C	D	α	β	γ	δ
014986	MRSA(R)	No	No	No	No	No	No	Yes	No
015386	MRSA(R)	No	No	No	No	Yes	No	No	Yes
015686	MRSA(R)	No	No	No	No	No	No	Yes	No
015786	MRSA(R)	Yes	No	Yes	No	Yes	Yes	No	Yes
018086	MRSA(R)	No	No	No	No	Yes	No	No	Yes
027186	MRSA(R)	Yes	No	No	No	Yes	No	Yes	No
027386	MRSA(R)	No	No	No	No	Yes	No	Yes	Yes
027586	MRSA(R)	No	No	No	No	Yes	No	Yes	Yes
027786	MRSA(R)	No	No	No	No	Yes	No	Yes	No
028186	MRSA(R)	No	Yes	Yes	No	Yes	No	Yes	Yes
028386	MRSA(R)	Yes	No	No	No	Yes	No	Yes	Yes
028486	MRSA(R)	No	No	No	No	Yes	No	Yes	No

Enterotoxin and Haemolysin production by isolates from different sources

Isolate	Collection	Enterotoxins				Haemolysins			
		A	B	C	D	α	β	γ	δ
028686	MRSA(R)	No	No	No	No	Yes	No	Yes	No
028786	MRSA(R)	No	Yes	Yes	No	Yes	No	Yes	No
028986	MRSA(R)	No	No	No	No	Yes	No	Yes	Yes
029286	MRSA(R)	No	No	No	No	Yes	No	Yes	Yes
029386	MRSA(R)	No	No	No	No	Yes	No	Yes	Yes
029586	MRSA(R)	No	No	No	No	Yes	No	Yes	No
029686	MRSA(R)	No	No	No	No	Yes	No	Yes	Yes
029886	MRSA(R)	Yes	Yes	Yes	No	No	Yes	Yes	Yes
030486	MRSA(R)	No	No	No	No	No	No	No	No

REFERENCES

Adlam, C, Easmon, C S F (1983) Immunity and hypersensitivity to staphylococcal infection. In *Staphylococci and staphylococcal infections, Vol. 1. Clinical and epidemiological aspects*, ed. Easmon, C S F & Adlam C, pp. 275-323. London: Academic Press Inc., Ltd.

Andrew, J H, Symons, D C (1982) Biotyping of MRSA as an epidemiological tool. *Abstract of Annual Scientific Meeting of the Australian Microbiological Society*, **11**;6.

Arbuthnott, J P (1983) Epidermolytic toxins. In *Staphylococci and staphylococcal infections, Vol. 2 The organism in vivo and in vitro*, ed. Easmon C S F & Adlam C, pp 599-617. London: Academic Press Inc., Ltd.

Archer, G L, Karchmer, A W, Vishniavsky, N, Johnston, L J (1984) Plasmid-pattern analysis for the differentiation of infecting from non-infecting *Staphylococcus epidermidis*. *Journal of Infectious Diseases*, **149**; 913-920.

Archer, G L, Pennell, E (1990) Detection of Methicillin Resistance in Staphylococci by using a DNA probe. *Antimicrobial Agents and Chemotherapy*, **34**; 1720-1724.

Arvidson, S O (1983) Extracellular enzymes from *Staphylococcus aureus*. In *Staphylococci and staphylococcal infections, Vol.2 The organism in vivo and in vitro*, ed. Easmon C S F & Adlam C, pp 745-808. London: Academic Press Inc., Ltd.

Bacon, A E, Jorgensen, K A, Wilson, K H, Kauffman, C A (1987) Emergence of nosocomial methicillin-resistant *Staphylococcus aureus* and therapy of colonized personnel during a hospital-wide outbreak. *Infection Control*, **8**;145-150.

Baird, D, Coia, J E (1987) Mupirocin-resistant *Staphylococcus aureus*. *Lancet*, **II**;387-388.

Baird-Parker, A C (1972) Classification and identification of staphylococci and their resistance to physical agents. In *The staphylococci*, ed. Cohen, J O, pp. 1-20. New York: John Wiley & Sons Inc.

Baldwin, D S, Levine, B B, McCluskey, R T, Gallo, G R (1968) Renal failure and interstitial nephritis due to penicillin and methicillin. *New England Journal of Medicine*, **279**;1245-1252.

Barber, M (1947) Staphylococcal infection due to penicillin-resistant strains. *British Medical Journal*, **2**;863-865.

Barber, M (1961) Methicillin-resistant staphylococci. *Journal of Clinical Pathology* **14**;385-393.

Barber, M, Rozwadowska-Dowzenko, M (1948) Infection by penicillin-resistant staphylococci. *Lancet*, **ii**;641-644.

Barrie, J D (1976) *Communicable Diseases Scotland weekly reports*, 76/37.

Bass, J W (1982) The spectrum of staphylococcal disease. From Job's boils to toxic shock. *Postgraduate Medicine*, **72**;58-75.

Bergdoll, M S (1983) Enterotoxins. In *Staphylococci and staphylococcal infections, Vol.2 The organism in vivo and in vitro*, ed. Easmon C S F & Adlam C, pp 559-598. London: Academic Press Inc., Ltd.

Bergdoll, M S (1985) The staphylococcal enterotoxins- an Update. In *The Staphylococci, Zentralblatt für Bakteriologie, Mikrobiologie und Hygiene* , ed. Jeljaszewicz J, Supplement 14 pp. 247-254. Stuttgart: Gustav Fischer Verlag.

Bergdoll, M S, Reiser, R F, Crass, B A, Robbins, R A (1985) The Toxic Shock Syndrome Toxin-One (TSST-1) and Toxic Shock Syndrome. In *The Staphylococci, Zentralblatt für Bakteriologie, Mikrobiologie und Hygiene* , ed. Jeljaszewicz J, Supplement 14 pp. 91-94. Stuttgart: Gustav Fischer Verlag.

Bergey's Manual of Determinative Bacteriology (1974) ed. Buchanan, R E, Gibbons, N E, 8th Edition, pp. 478-489. Baltimore: The Williams and Wilkins Company.

Birnboim, H C, Doly, J (1979) A rapid, alkaline extraction procedure for screening recombinant DNA. *Nucleic Acids Research*, **7**;1513-1523.

Bjorvatn, B, Lund, V, Kristiansen, B-E, Korsnes, L, Spanne, O, Lindquist, B (1984) Applications of restriction endonuclease fingerprinting of chromosonal DNA of *Neisseria meningitidis*. *Journal of Clinical Microbiology*, **19**; 763-765.

Bradbury, W C, Pearson, A D, Marko, M A, Corgi, R V, Penner, J L (1984) Investigation of a *Campylobacter jejuni* outbreak by serotyping and chromosonal restriction endonuclease analysis. *Journal of Clinical Microbiology*, **19**;342-346.

Branger, C, Goullet, Ph (1987) Esterase electrophoretic polymorphism of methicillin-sensitive and methicillin-resistant strains of *Staphylococcus aureus*. *Journal of Medical Microbiology*, **24**; 275-281.

Branger, C, Goullet, Ph (1989) Genetic heterogeneity in methicillin-resistant strains of *Staphylococcus aureus* revealed by esterase electrophoretic polymorphism. *Journal of Hospital Infection*, **14**; 125-134.

Broda, P (1979) *Plasmids*. Oxford: W H Freeman and Co.

Burnette, W N (1981) "Western Blotting": electrophoretic transfer of proteins from sodium dodecyl sulphate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Analytical Biochemistry*, **112**;195-203.

Burnie, J P, Lee, W, Matthews, R C, Bayston, R (1988) Immunoblot fingerprinting of coagulase negative staphylococci. *Journal of Clinical Pathology*, **41**;103-110.

Burnie, J P, Matthews, R C (1987) Immunoblot analysis: a new method for fingerprinting hospital pathogens. *Journal of Immunological Methods*, **100**;41-46.

Burnie, J P, Matthews, R C, Lee, W, Murdoch, D (1989) A comparison of immunoblot and DNA restriction patterns in characterising methicillin- resistant isolates of *Staphylococcus aureus*. *Journal of Medical Microbiology*, **29**; 255-261.

Cafferkey, M T, Coleman, D C, McGrath, B, Hone, R, Pomeroy, H, Ruddy, R, Keane C T (1985) Methicillin-resistant *Staphylococcus aureus* in Dublin 1971-1984. *Lancet*, **ii**;705-708.

Cafferkey, M T, Hone, R, Falkiner, F R, Keane, C T, Pomeroy, H (1983) Gentamicin and methicillin resistant *Staphylococcus aureus* in Dublin hospitals: clinical and laboratory studies. *Journal of Medical Microbiology*, **16**;117-127.

Cafferkey, M T, Hone, R, Keane, C T (1988) Sources and outcome for methicillin-resistant *Staphylococcus aureus* bacteraemia. *Journal of Hospital Infection*. **11**;136-143.

Canepari, P, Varaldo, P E, Fontana R, Satta G (1985) Different staphylococcal species contain various numbers of penicillin-binding proteins ranging from four (*Staphylococcus aureus*) to only one (*Staphylococcus hycius*). *Journal of Bacteriology*, **163**;796-798.

Carroll, J D, Pomeroy, H M, Russell, R J, Arbuthnott, J P, Keane, C T, McCormick, O M, Coleman, D C (1989) A new methicillin- and gentamicin-resistant *Staphylococcus aureus* in Dublin: molecular genetic analysis. *Journal of Medical Microbiology*, **28**;15-23.

Casewell, M W (1986) Epidemiology and control of the 'modern' methicillin-resistant *Staphylococcus aureus*. *Journal of Hospital Infection*, **7** Supplement A;1-11.

Chao, L P, Birkbeck, T H (1978) Assay of staphylococcal delta-haemolysin with fish erythrocytes. *Journal of Medical Microbiology*, **11**;303-313.

Christensson, B, Hedström, S A (1986) Biochemical and biological properties of *Staphylococcus aureus* septicaemia strains in relation to clinical characteristics. *Scandinavian Journal of Infectious Diseases*, **18**;297-303.

Coia, J E, Noor-Hussain, I, Platt, D J (1988) Plasmid profiles and restriction enzyme fragmentation patterns of plasmids of methicillin-sensitive and methicillin-resistant isolates of *Staphylococcus aureus* from hospital and the community. *Journal of Medical Microbiology*, **27**;271-276.

Coia, J E, Thomson-Carter, F, Baird, D, Platt, D J (1990) Characterisation of methicillin-resistant *Staphylococcus aureus* by biotyping, immunoblotting and restriction enzyme fragmentation patterns. *Journal of Medical Microbiology*, **31**;125-132.

Communicable Diseases (Scotland) Unit (1977) *Communicable Diseases Scotland weekly reports*, 77/24.

Cooke, E M, Casewell, M W, Emmerson, A M, Gaston, M, de Saxe, M, Mayon-White, R T, Galbraith, N S (1986) Methicillin-resistant *Staphylococcus aureus* in the UK and Ireland. A questionnaire survey. *Journal of Hospital Infection*, **8**;143-148.

Cookson, B, Talsania, H, Naidoo, J, Phillips, I (1986) Strategies for typing and properties of epidemic methicillin-resistant *Staphylococcus aureus*. *European Journal of Clinical Microbiology*, **5**; 702-709.

Crass, B A, Bergdoll, M S (1986) Involvement of staphylococcal enterotoxins in nonmenstrual toxic shock syndrome. *Journal of Clinical Microbiology*, **23**;1138-1139.

Cutler, R R (1979) Relationship between antibiotic resistance, the production of "virulence factors", and virulence for experimental animals in *Staphylococcus aureus*. *Journal of Medical Microbiology*, **19**;55-62.

Dacre, J, Emmerson, A M, Jenner, E A (1986) Gentamicin-methicillin-resistant *Staphylococcus aureus*: epidemiology and containment of an outbreak. *Journal of Hospital Infection*, **7**;130-136.

Datta, N (1977) Classification of R-plasmids. In *R factor Drug Resistance Plasmids*, ed. Mitsuhashi, S, pp 255-272. Baltimore: University Park Press.

Devriese, L A, Oeding, P (1976) Characteristics of *Staphylococcus aureus* strains isolated from different animal species. *Research in Veterinary Science*, **21**;284-291.

Dice, L R (1945) Measures of the amount of ecologic association between species. *Ecology*, **26**;297-302.

Dowd, G, Cafferkey, M T, Dougan, G (1983) Gentamicin and methicillin-resistant *Staphylococcus aureus* in Dublin hospitals; molecular studies. *Journal of Medical Microbiology*, **16**; 129-138.

Duckworth, G J, Lothian, J L E, Williams, J D (1988) Methicillin-resistant *Staphylococcus aureus*: report of an outbreak in a London teaching hospital. *Journal of Hospital Infection*, **11**;1-15.

Duckworth, G J, Oppenheim, B A (1986) Enterotoxin production in epidemic methicillin-resistant *Staphylococcus aureus*. *Lancet*, **1**;565-566.

Duguid, J P (1989) *Staphylococcus*: cluster-forming Gram-positive cocci. In *Mackie & McCartney Practical Medical Microbiology*, ed. Collee, J G, Duguid, J P, Fraser, A G, Marmion, B P, pp. 303-316. Edinburgh: Churchill Livingstone.

Elek, S D, Moryson, C (1974) Resistotyping of *Staphylococcus aureus*. *Journal of Medical Microbiology*, **7**;237-251.

Emslie, K R, Townsend, D E, Grubb, W B (1985) A resistance determinant to nucleic acid binding compounds in methicillin-resistant *Staphylococcus aureus*. *Journal of Medical Microbiology*, **20**;139-148.

Fackrell, H B, Austin, J W, Simpson, D A (1985) Initial characterisation of the interaction of staphylococcal α-toxin with its receptor on erythrocyte membranes. In *The Staphylococci, Zentralblatt für Bakteriologie, Mikrobiologie und Hygiene* , ed. Jeljaszewicz J, Supplement 14 pp. 287-293. Stuttgart: Gustav Fischer Verlag.

Farrar, W E (1983) Molecular analysis of plasmids in epidemiologic investigation. *Journal of Infectious Diseases*, **148**;1-6.

Fisk, R T (1942a) Studies on staphylococci I. Occurence of bacteriophage carriers among strains of *Staphylococcus aureus*. *Journal of Infectious Diseases*, **71**;153-160.

Fisk, R T (1942b) Studies on staphylococci II. Identification of *Staphylococcus aureus* strains by means of bacteriophage. *Journal of Infectious Diseases*, **71**;161-165.

Forbes, B A, Schaberg, D R (1983) Transfer of resistance plasmids from *Staphylococcus epidermidis* to *Staphylococcus aureus*: evidence for conjugative exchange of resistance. *Journal of Bacteriology*, **153**;627-634.

Forsgren, A, Ghetie, V, Lindmark R, Sjöquist, J (1983) Protein A and its exploitation. In *Staphylococci and staphylococcal infections, Vol.2 The organism in vivo and in vitro*, ed. Easmon C S F & Adlam C, pp 429-480. London: Academic Press Inc., Ltd.

Freer, J H, Birkbeck, T H (1982) Possible conformation of delta-lysin, a membrane-damaging peptide of *Staphylococcus aureus*. *Journal of Theoretical Biology*, **94**;535-540.

French, G L, Cheng, A F, Ling, J M, Mo, P, Donnan, S (1990) Hong Kong strains of methicillin-resistant and methicillin-sensitive *Staphylococcus aureus* have similar virulence. *Journal of Hospital Infection*, **15**;117-125.

Gaston, M A, Duff, P S, Naidoo, J, Ellis, K, Roberts, J I S, Richardson, J F, Marples, R R, Cooke, E M (1988) Evaluation of electrophoretic methods for typing methicillin-resistant *Staphylococcus aureus*. *Journal of Medical Microbiology*, **26**;189-197.

Gelmi, M, Foresti, I, Ravizzola, G, Bonfanti, C, Verardi, R, Caruso, A, Turano, A (1987) Antibiotic resistances and plasmids in *Staphylococcus aureus* from Italian hospitals. *Journal of Medical Microbiology*, **23**;111-118.

Gill, P, Jeffreys, A J, Werrett, D J (1985) Forensic application of DNA "fingerprints". *Nature*, **318**;577-579.

Gillespie, M T, Lyon, B R, Skurray, R A (1990) Typing of methicillin-resistant *Staphylococcus aureus* by antibiotic resistance phenotypes. *Journal of Medical Microbiology*, **31**;57-64.

Goldmann, D A (1987) New Microbiological Techniques for Hospital Epidemiology. *European Journal of Clinical Microbiology*, **6**;344-347.

Grimont, F, Chevrier, D, Grimont, P A, Lefevre, M, Guesdon, J L (1989) Acetylaminofluorene-labelled ribosomal RNA for use in molecular epidemiology and taxonomy. *Research Microbiology*, **140**;447-454.

Grimont, F, Grimont, P A (1986) Ribosomal ribonucleic acid gene restriction patterns as potential taxonomic tools. *Annals of the Institute Pasteur Microbiology*, **137b**;165-175.

Grubb, W B, Townsend, D E, Ashdown, N, Taheri, S (1985) A genetic comparison of methicillin-resistant *Staphylococcus aureus* isolated from different countries. *Proceedings of the 14th International Congress of Chemotherapy*, Kyoto 23-28 June.

Hajek, V, Marsalek, E (1971) Differenzierueng pathogener staphylokokken und vorschlag für ihre taxonomische klassifikation. *Zentralblatt für Bakteriologie, I. Abteilung Orig. A* **217**;176-182.

Hammond, S M, Lambert, P A (1981) *Antibiotics and Antimicrobial Action*, pp 22-25. London: Edward Arnold.

Hawkey, P M (1987) Molecular methods for the investigation of bacterial cross-infection. *Journal of Hospital Infection*, **9**;211-218.

Humphreys, H, Carroll, J D, Keane, C T, Cafferkey, M T, Pomeroy, H M, Coleman, D C (1990) Importation of methicillin-resistant *Staphylococcus aureus* from Baghdad to Dublin and subsequent nosocomial spread. *Journal of Hospital Infection*, **15**;127-135.

Humphreys, H, Keane, C T, Hone, R, Pomeroy, H M, Russell, R J, Arbuthnott, J P, Coleman, D C (1989) Enterotoxin production by *Staphylococcus aureus* isolates from cases of septicaemia and from healthy carriers. *Journal of Medical Microbiology*, **28**;163-172.

Hunter, P R, Gaston, M A (1988) Numerical Index of the Discriminatory Ability of Typing Systems: an application of Simpson's Index of Diversity. *Journal of Clinical Microbiology*, **26**;2465-2466.

Irino, K, Grimont, F, Casin, I, Grimont, P A (1988) rRNA gene restriction patterns of *Haemophilus influenzae* biogroup *aegyptius* strains associated with Brazilian purpuric fever. *Journal of Clinical Microbiology*, **26**;1535-1538.

Jepsen, O B (1986) The demise of the 'old' methicillin-resistant *Staphylococcus aureus*. *Journal of Hospital Infection* **7** Supplement A;13-17.

Jevons M P (1961) "Celbenin"-resistant staphylococci. *British Medical Journal* **1**;124-125.

Jevons, M P, Parker, M T (1964) The evolution of new hospital strains of *Staphylococcus aureus*. *Journal of Clinical Pathology*, **17**;243-250.

John, J F, McKee, K T, Twitty, J A, Schaffner, W (1983) Molecular epidemiology of sequential nursery epidemics caused by multiresistant *Klebsiella pneumoniae*. *Journal of Paediatrics*, **102**;825-830.

Jordens, J Z, Duckworth, G J, Williams, R J (1989) Production of "virulence factors" by "epidemic" methicillin-resistant *Staphylococcus aureus in vitro*. *Journal of Medical Microbiology*, **30**;245-252.

Jordens, J Z, Hall, L M C (1988) Characterisation of methicillin-resistant *Staphylococcus aureus* isolates by restriction endonuclease digestion of chromosomal DNA. *Journal of Medical Microbiology*, **27**;117-123.

Kaplan, M H, Tenenbaum, M J (1982) *Staphylococcus aureus*: cellular biology and clinical application. *American Journal of Medicine*, **72**;248-258.

Kass, E H, Parsonnet, J (1987) On the pathogenesis of toxic shock syndrome. *Reviews of Infectious Diseases*, **9** Supplement 5;482-489.

Kayser, F H (1975) Methicillin-resistant staphylococci 1965-75. *Lancet*, **ii**;650-652.

Kayser, F H, Berger-Bachi, B, Beck, W D (1986) Genetics of multiply-resistant *Staphylococcus aureus*. *Journal of Hospital Infection*, **7** Supplement A;19-27.

Kayser, F H, Mak, T M (1972). Methicillin-resistant staphylococci. *American Journal of Medical Science*, **264**;197-205.

Knox R, (1960). A new penicillin (BRL 1241) active against penicillin-resistant staphylococci. *British Medical Journal*, **2**;690-693.

Knudsen, E T, Rolinson, G N (1960). Absorption and excretion of a new antibiotic (BRL 1241). *British Medical Journal*, **2**;700-703.

Krikler, S J, Pennington, T H, Petrie, D (1986) Typing of strains of *Staphylococcus aureus* by Western blot analysis of culture supernates. *Journal of Medical Microbiology*, **21**;169-171.

Kucers, A, Bennett, N McK (1989) Vancomycin. In *The Use of Antibiotics*, ed. Kucers A & Bennett, N McK, 4th Edition, pp. 1045-1068. Oxford: Heinemann Press.

Kuhl, S A, Patee, P A, Baldwin, J N (1978) Chromosomal map location of the methicillin resistance determinant in *Staphylococcus aureus*. *Journal of Bacteriology*, **135**;460-465.

Lacey, R W (1972) Genetic control in methicillin-resistant strains of *Staphylococcus aureus*. *Journal of Medical Microbiology*, **5**;497-508.

Lacey, R W (1975) Antibiotic resistance plasmids of *Staphylococcus aureus* and their clinical importance. *Bacteriological Reviews*, **39**; 1-32.

Lacey, R W (1987) Multi-resistant *Staphylococcus aureus*- a suitable case for inactivity. *Journal of Hospital Infection*, **9**;103-105.

Lacey, R W, Barr, K W, Barr, V E, Inglis, T J (1986) Properties of methicillin-resistant *Staphylococcus aureus* colonizing patients in a burns unit. *Journal of Hospital Infection*, **7**;137-148.

Lacey, R W, Chopra, I (1975) Effect of plasmid carriage on the virulence of *Staphylococcus aureus*. *Journal of Medical Microbiology*, **8**;137-147.

Lacey, R W, Grinsted, J (1973) Genetic analysis of methicillin-resistant *Staphylococcus aureus*; Evidence for their evolution from a single clone. *Journal of Medical Microbiology*, **6**;511-526.

Lacey, R W, Stokes, A (1979) Studies on recently isolated cultures of methicillin-resistant *Staphylococcus aureus*. *Journal of General Microbiology*, **114**;329-339.

Laemmli, U K (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**;680-685.

Langenberg, W, Rouws, E A J, Widjojokusumo, A, Tygat, G N J, Zanen, H C (1986) Identification of *Campylobacter pyloridis* isolates by restriction endonuclease DNA analysis. *Journal of Clinical Microbiology*, **24**; 414-417.

Lee, W, Burnie, J P (1988) Fingerprinting methicillin-resistant *Staphylococcus aureus* by the immunoblot technique. *Journal of Medical Microbiology*, **25**; 261-268.

Lee, W, Burnie, J P, Matthews, R C (1986) Fingerprinting *Candida albicans*. *Journal of Immunological Methods*, **93**; 177-182.

Locksley, R M, Cohen, M L, Quinn, T C, Tompkins, L S, Coyle, M B, Kirihara, J M, Counts, G W (1982) Multiply antibiotic-resistant *Staphylococcus aureus*: introduction, transmission and evolution of nosocomial infection. *Annals of Internal Medicine* **97**;317-324.

Lyon, B R, Skurray R (1987) Antimicrobial Resistance of *Staphylococcus aureus*: Genetic Basis. *Microbiological Reviews*, **51**;88-134.

Mandell, G L (1975) Catalase, superoxide dismutase, and virulence of *Staphylococcus aureus*. *In vitro* and *in vivo* studies with emphasis on staphylococcal-leukocyte interaction. *Journal of Clinical Investigation*, **55**;561-566.

Maple, P A, Hamilton-Miller, J M, Brumfitt, W (1989) World-wide antibiotic resistance in methicillin-resistant *Staphylococcus aureus*. *Lancet*, **1**;537-540.

Marples R R, Cooke E M (1988) Current problems with methicillin-resistant *Staphylococcus aureus*. *Journal of Hospital Infection*, **11**;381-392.

Marples R R, Richardson J F, de Saxe M J (1986) Bacteriological characters of strains of *Staphylococcus aureus* submitted to a reference laboratory related to methicillin resistance. *Journal of Hygiene*, **96**;217-223.

Marples, R R (1988) Methicillin-resistant *Staphylococcus aureus*. *Current opinion in Infectious diseases*, **1**;722-726.

McKay, I, Coia, J E, Poxton I (1989) Typing of *Clostridium difficile* causing diarrhoea in an orthopaedic ward. *Journal of Clinical Pathology*, **42**; 511-515.

Mekalanos, J J (1983) Duplication and amplification of toxin genes in *Vibrio cholerae*. *Cell*, **35**;253-263.

Melish, M E, Glasgow, L A (1970) The staphylococcal scalded skin syndrome: development of an experimental model. *New England Journal of Medicine*, **282**;1114-1119.

Möllby, R (1983) Isolation and properties of membrane damaging toxins. In *Staphylococci and staphylococcal infections, Vol. 2 The organism in vivo and in vitro*, ed. Easmon C S F & Adlam C, pp 619-669. London: Academic Press Inc., Ltd.

Morgan, M G, Harte-Barry, M J (1989) Methicillin-resistant *Staphylococcus aureus*: a ten-year survey in a Dublin hospital. *Journal of Hospital Infection*, **14**;357-362.

Musher, D M, Verner, E F (1985) Treatment of Infections due to *Staphylococcus aureus*. In *The Staphylococci, Zentralblatt für Bakteriologie, Mikrobiologie und Hygiene* , ed. Jeljaszewicz J, Supplement 14 pp. 407-419. Stuttgart: Gustav Fischer Verlag.

Noble, W C, White, M I (1983) Staphylococcal skin infections in man. In *Staphylococci and staphylococcal infections, Vol. 1. Clinical and epidemiological aspects*, ed. Easmon, C S F & Adlam C, pp.165-192. London: Academic Press Inc., Ltd.

Novick, R P (1980) Plasmids. *Scientific American*, **243**; 76-90.

Oeding, P (1983) Taxonomy and identification. In *Staphylococci and staphylococcal infections, Vol. 1. Clinical and epidemiological aspects*, ed. Easmon, C S F & Adlam C, pp. 1-31. London: Academic Press Inc., Ltd.

Ogston, A (1881) Report upon micro-organisms in surgical diseases. *British Medical Journal*, 1;369-375.

Parker M T (1983). The significance of phage-typing patterns in *Staphylococcus aureus*. In *Staphylococci and staphylococcal infections, Vol. 1. Clinical and epidemiological aspects*, ed. Easmon, C S F & Adlam C, pp. 33-62. London: Academic Press Inc., Ltd.

Parker, M T, Hewitt, J H (1970) Methicillin resistance in *Staphylococcus aureus*. *Lancet*, **1**;800-804.

Pavillard, R, Harvey, K, Douglas, D, Hewstone, A, Andrew, J, Collopy, B, Ashe, V, Carson, P, Davidson, A, Gilbert, G, Spicer, J, Tosolini, G (1982) Epidemic of hospital-acquired infection due to methicillin-resistant *Staphylococcus aureus* in major Victorian hospitals. *Medical Journal of Australia*, **1**;451-454.

Peacock, J E, Moorman, D R, Wenzel, R P, Mandell, G L (1981) Methicillin-resistant *Staphylococcus aureus*: microbiologic characteristics, antimicrobial susceptibilities, and assessment of virulence of an epidemic strain. *Journal of Infectious Diseases*, **144**;575-582.

Pearman, J W, Christensen, K J, Annear, D I, Goodwin, C S, Metcalf, C, Donovan, F P, Macey, K L, Bassette, L D, Powell, I M, Green, J M, Harper, W E, McKelvie, M S (1985) Control of methicillin-resistant *Staphylococcus aureus* (MRSA) in an Australian metropolitan teaching hospital complex. *Medical Journal of Australia*, **142**;103-108.

Platt, D J (1983) Bacterial plasmids and their fingerprints. *Disease Markers*, **1**; 107-115.

Platt, D J (1987) A simple statistical approach that represents the frequency distribution of plasmids in clinical isolates of the enterobacteria. *Journal of Medical Microbiology*, **23**;255-260.

Platt, D J, Brown, D J, Munro, D S (1986a) The distribution of plasmids among a representative collection of Scottish strains of salmonellae. *Journal of Hygiene*, **97**;199-204.

Platt, D J, Chesham, J S, Brown, D J, Kraft, C A, Taggart, J (1986b) Restriction enzyme fingerprinting of enterobacterial plasmids: a simple strategy with wide application. *Journal of Hygiene*, **97**; 205-210.

Platt, D J, Sommerville, J S (1981) A simple method for the detection of resistance plasmids in *Serratia* species by transfer to members of the genus *Enterobacter*. *Journal of Antimicrobial Chemotherapy*, **8**; 145-152.

Platt, D J, Sommerville, J S, Kratt, C A, Timbury M C (1984) Antimicrobial resistance and the ecology of *Escherichia coli* plasmids. *Journal of Hygiene*, **93**;181-188.

Platt, D J, Taggart, J (1987) Molecular epidemiology: Determination of plasmid sizes. *Focus*, **9**;3, 13

Plikaytis, B D, Carlone, G M, Edmonds, P, Mayer, L W (1986) Robust estimation of standard curves for protein molecular weight and linear-duplex DNA base-pair number after gel electrophoresis. *Analytical Biochemistry*, **152**;346-364.

Plorde, J J, Sherris, J C (1974) Staphylococcal resistance to antibiotics: origin, measurement and epidemiology. *Annals of the New York Academy of Sciences*, **236**;413-434.

Preheim, L C, Rimland D, Bittner, M J (1987) Methicillin-resistant *Staphylococcus aureus* in Veterans Administration Medical Centers. *Infection Control*, **8**;191-194.

Rhinehart, E, Shlaes, D M, Keys, T F, Serkey, J, Kirkley, B, Kim, C, Currie-McCumber, C A, Hall, G (1987) Nosocomial clonal dissemination of methicillin-resistant *Staphylococcus aureus*: Elucidation by plasmid analysis. *Archives of Internal Medicine*, **147**; 521-524.

Richardson, J F, Chittasobhon, N, Marples, R R (1988) Supplementary phages for the investigation of strains of methicillin-resistant *Staphylococcus aureus*. *Journal of Medical Microbiology*, **25**;67-74.

Roberts, J I S, Gaston, M A (1987) Protein A and coagulase expression in epidemic and non-epidemic *Staphylococcus aureus*. *Journal of Clinical Pathology*, **40**;837-840.

Rountree P M (1978). History of staphylococcal infection in Australia. *Medical Journal of Australia*, **2**;543-546.

Schaberg, D R, Zervos, M (1986) Plasmid analysis in the study of the epidemiology of nosocomial Gram-positive cocci. *Reviews of Infectious Diseases*, **8**;705-712.

Scottish MRSA Study Group (1987) MRSA IN SCOTLAND: First report of the Scottish MRSA Study Group. *Communicable Diseases Scotland weekly reports*, 87/51.

Scottish MRSA Study Group (1988) MRSA IN SCOTLAND: Second report of the Scottish MRSA Study Group. *Communicable Diseases Scotland weekly reports*, 88/29.

Shalita, Z, Murphy, E, Novick, R P (1980) Penicillinase plasmids of *Staphylococcus aureus*: structural and evolutionary relationships. *Plasmid*, **3**;291-311.

Shanson, D C (1981). Antibiotic-resistant *Staphylococcus aureus*. *Journal of Hospital Infection*, **2**:11-36.

Shanson, D C (1986) Staphylococcal infections in hospital. *British Journal of Hospital Medicine*, **35**;312-320.

Siegel, S (1956) *Non-parametric statistics for the behavioural sciences*, pp. 104-111. London: McGraw-Hill.

Sjöström, J E, Löfdahl, S, Philipson, L (1975) Transformation reveals a chromosomal locus of the gene(s) for methicillin-resistance in *Staphylococcus aureus*. *Journal of Bacteriology*, **123**;905-915.

Southern, E M (1975) Detection of specific sequences among DNA fragments separated by gel electorphoresis. *Journal of Molecular Biology*, **98**; 503-517.

Spink, W W, Ferris, V (1945) Quantitative action of penicillin inhibitor from penicillin-resistant strains of staphylococci. *Science*, **102**;221-223.

Stephenson, J R, Cook, S J, Tabaqchali, S (1986) New method for typing *Staphylococcus aureus* resistant to methicillin based on sulphur-35 methionine labelled proteins: its application in an outbreak. *British Medical Journal*, **293**; 581-583.

Taylor, D N, Wachsmuth, K, Shangkuan, Y-H, Schmidt, E V, Barrett, T S, Schrader, J S, Scherach, C S, McGee, H B, Feldman, R A, Brenner, D J (1982) Salmonellosis associated with marijuana. *New England Journal of Medicine*, **306**; 1249-1253.

Thelestam, M (1983) Modes of membrane damaging action of staphylococcal toxins. In *Staphylococci and staphylococcal infections, Vol. 2 The organism in vivo and in vitro*, ed. Easmon C S F & Adlam C, pp 705-744. London: Academic Press Inc., Ltd.

Thomson-Carter, F M, Pennington, T H (1989) Characterisation of methicillin-resistant isolates of *Staphylococcus aureus* by analysis of whole-cell and exported proteins. *Journal of Medical Microbiology*, **28**; 25-32.

Threlfall, E J, Hall, M L M, Rowe, B (1986) *Salmonella gold-coast* from outbreaks of food-poisoning in the British Isles can be differentiated by plasmid profiles. *Journal of Hygiene*, **97**;115-122.

Towbin, H, Stachelin, T, Gordon, J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the USA*, **76**; 4350-4354.

Townsend, D E, Ashdown, N, Bolton, S, Bradley, J, Duckworth, G, Moorhouse, E C, Grubb, W B (1987) The international spread of methicillin-resistant *Staphylococcus aureus*. *Journal of Hospital Infection*, **9**;60-71.

Townsend, D E, Ashdown, N, Greed, L C, Grubb, W B (1984) Analysis of plasmids mediating gentamicin resistance in methicillin-resistant *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy*, **13**;347-352.

Townsend, D E, Ashdown, N, Momoh, M, Grubb, W B (1985a) Distribution of plasmid-borne resistance to nucleic acid binding compounds in methicillin-resistant *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy*, **15**;417-434.

Townsend, D E, Ashdown, N, Pearman, J W, Annear, D I, Grubb, W B (1985b) Genetics and epidemiology of methicillin-resistant *Staphylococcus aureus* isolated in a Western Australian hospital. *Medical Journal of Australia*, **142**;108-111.

Townsend, D E, Bolton, S, Ashdown, N, Grubb, W B (1985c) Transfer of plasmid-borne aminoglycoside resistance determinants in staphylococci. *Journal of Medical Microbiology*, **20**; 169-185.

Turnidge, J, Lawson, P, Munro, R, Benn, R (1989) A national survey of antimicrobial resistance in *Staphylococcus aureus* in Australian teaching hospitals. *Medical Journal of Australia*, **150**;69-72.

Van Ketel, R J, ter Schegget, J, Zanen, H C (1984) Molecular epidemiology of *Legionella pneumophila* serogroup 1. *Journal of Clinical Microbiology*, **20**;362-364.

Vickery, A M, Beard-Pegler, M A, Rountree, P M (1988) Strain differentiation in methicilin-resistant *Staphylococcus aureus*. *Pathology*, **15**;235-240.

Vickery, A M, Beard-Pegler, M A, Stubbs, E (1986) Phage-typing patterns and lysogenicity of methicillin-resistant strains of *Staphylococcus aureus* from Sydney, Australia, 1965-85. *Journal of Medical Microbiology*, **22**;209-216.

Wachsmuth, K (1986) Molecular epidermiology of bacterial infections: examples of methodology and of investigations of outbreaks. *Reviews of Infectious Diseases*, **8**; 682-692.

Wadström, T (1983) Biological effects of cell damaging toxins. In *Staphylococci and staphylococcal infections, Vol. 2 The organism in vivo and in vitro*, ed. Easmon C S F & Adlam C, pp 671-704. London: Academic Press Inc., Ltd.

Waldvögel, F A (1986) Treatment of infections due to methicillin-resistant *Staphylococcus aureus*. *Journal of Hospital Infection*, **7** Supplement A;37-46.

White, A B (1982) *Communicable Diseases Scotland weekly reports*, 82/11.

Wiley, B B, Rogolsky, M S (1985) Phospholipase activity associated with electrofocussed staphylococcal exfoliative toxin (ET). In *The Staphylococci, Zentralblatt für Bakteriologie, Mikrobiologie und Hygiene* , ed. Jeljaszewicz J, Supplement 14 pp. 295-300. Stuttgart: Gustav Fischer Verlag.

Wilkinson, B J (1983) Staphylococcal capsules and slime. In *Staphylococci and staphylococcal infections, Vol. 2 The organism in vivo and in vitro*, ed. Easmon C S F & Adlam C, pp 481-523. London: Academic Press Inc., Ltd.

Wilson, G S, Atkinson, J D (1945) Typing of staphylococci by bacteriophage method. *Lancet*, **1**;647-648.

Wyke, A W (1984) Isolation of five penicillin-binding proteins from *Staphylococcus aureus*. *FEMS Microbiology Letters*, **22**;133-138.