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Population biology of *Staphylococcus aureus* on dairy cattle farms

by

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biological Sciences

University of Warwick, Department of Biological Sciences

March 2004

for my parents and grandparents

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Acknowledgements

In completing this thesis I owe a debt of gratitude to a great number of people for their help, encouragement and advice over the past few years. Whilst it would be impossible to mention everyone by name, the people mentioned below deserve special thanks.

First and foremost, I extend my gratitude to my supervisors (both official and surrogate) Drs. Laura Green, Graham Medley and Crawford Dow, and Professor Chris Dowson. They have been a continual source of ideas and inspiration, and their guidance has been invaluable.

In addition I thank the members, past and present, of the Ecology and Epidemiology, Infectious Disease and Molecular Physiology Groups; and the backroom people, Jane Green and Vicky Cooper in the lab. Dee King in the library, and John, Tim and Bob in stores who have all helped to make things run smoothly.

For contributions both intellectual and bacteriological I would like to thank (in no particular order) Dr. Andrew Bradley, Martin Green, Professor Ynte Schukken, Dr. Larry Fox, Dr. Nestor Tadich and Dr. Juan Kruze.

Without the access to instruments and machines permitted by Drs Dave Scanlan and Donna Slater, much of the work presented in this thesis would never have been completed, and so I am truly grateful to them. In addition I would like to thank the Molecular Biology Service, in particular Dr. Helen Bird, for their efforts and always managing to force a smile when I appeared with another two weeks work.

In Somerset, I extend my gratitude to Steve, Clive and Judith and their respective families for allowing me access to their farm and animals, and for providing me with a place to stay when I visited. I am also grateful to Allan and Kelly, and the cows who put up with my visits without too much complaint.

I would also like to thank the BBSRC for funding this work.

I have to mention Julia who has put up with a lot over the past few years. Finally I must acknowledge my Mum and Dad, who have unquestioningly supported me in everything I have chosen to do, and are always behind me at every turn.

Thank you to you all, and to everyone who wasn't mentioned individually.

Declaration

I declare that the work presented in this thesis is my own, with the caveats outlined below, and has not been submitted for a degree at another university.

A section of the work presented in Chapter 2 has been published (below) and a reprint of this publication is presented in Appendix 1:
Smith, E.M., Green, L.E., Mason, D. (2003). Savinase is a bactericidal enzyme. *Applied and Environmental Microbiology*, **69** (1), 719-721.

The Fox collection of isolates analysed [Chapter 5] was provided by Y.H. Schukken, and were first published in:
Fox, L.K., Gershman, M., Hancock, D.D., Hutton, C.T. (1991). Fomites and reservoirs of *Staphylococcus aureus* causing intramammary infections as determined by phage typing: The effect of milking time hygiene practices. *Cornell Veterinarian*, **81**, 183-193.

Chilean and UK *Staphylococcus aureus* isolates were provided by Drs. N. Tadich and J. Kruze (University of Austral, Chile) and Dr. A. J. Bradley (University of Bristol) respectively. Martin Green (University of Warwick) provided raw milk samples for analysis.

Pulsed-field gel electrophoresis results discussed in Chapter 5 were provided by Y.H. Schukken, and have been published in:
Zadoks, R.N., van Leeuwen, W.B., Kreft, D., Fox, L.K., Barkema, H.W., Schukken, Y.H., van Belkum, A. (2002). Comparison of *Staphylococcus aureus* isolates from bovine and human skin, milking equipment, and bovine milk by phage typing, pulsed-field gel electrophoresis and binary typing. *Journal of Clinical Microbiology*, **40** (11), 3894-3902.

Edward M. Smith
(March, 2004)

Abstract

A total of 450 isolates of *Staphylococcus aureus* were typed using multilocus sequence typing (MLST). They were isolated from the cattle and environment of a UK organic dairy farm, 43 dairy herds in the USA, the mammary glands of cattle in Chile and the UK, and also included the reference strain Newbould 305 (NCIMB 702892). Strains were compared to investigate differences between isolates from varying sites of isolation and the population detected on a single farm.

MLST was suitable for the differentiation of bovine associated *S. aureus*, and thirty different sequence types were detected. These contained a number of novel alleles detected at each loci, agreeing with previous reports of host-specialisation, and indicating localised strain evolution. MLST was also able to discriminate between isolates detected in milk and on teat skin. The Newbould 305 strain was significantly ($p<0.05$) associated with teat skin, and this may have important implications for future studies. The majority of isolates (87.4%) were present within one previously undescribed clonal complex (CC97), which contained representatives from all three geographic locations.

Analysis of isolates from a single farm demonstrated the clonality of the organism, supporting the theory of cow-to-cow spread of disease. No isolates were detected in heifer mammary secretions, suggesting transmission to these animals did not occur. Typing of multiple colonies from a single sample demonstrated strain heterogeneity within individual quarters. Environmental isolates were detected, though it is unlikely that they formed a significant reservoir of infection on the farm studied.

Abbreviations

ABI	Applied Biosystems
AP	allelic profile
AP-PCR	arbitrarily primed PCR
BD	Becton Dickinson
BMSCC	bulk milk SCC
bp	base pairs
Bronopol	2-bromo-2-nitropropane-1,3-diol
BSA	bovine serum albumin
BT	binary typing
BURST	based upon related sequence types
°C	degrees centigrade
C.	*Corynebacterium*
χ^2	Chi-squared
CC	clonal complex
CCD	charge coupled device
CDC	Centers for disease control and prevention
cfu	colony forming units
CI	confidence interval
CM	clinical mastitis
cm	centimetre
CNS	coagulase-negative staphylococci
CPS	coagulase-positive staphylococci
D	Simpson's index of diversity
dATP	deoxyadenosine triphosphate
DCT	dry cow therapy
dCTP	deoxycytidine triphosphate
Dec	December
dGTP	deoxyguanosine triphosphate
DIM	days in milk
DLV	double locus variant
DMSO	dimethyl sulfoxide

d_N	number of non-synonymous changes
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
d_S	number of synonymous changes
dTTP	deoxythymidine triphosphate
E.	*Escherichia*
eBURST	enhanced BURST
EDTA	ethylenediaminetetraacetic acid
FAFLP	fluorescent amplified fragment length polymorphism
FCM	flow cytometry
Feb	February
Fig.	figure
FISH	fluorescent *in situ* hybridisation
FITC	fluorescein isothiocyanate
FSC	forward scatter
$\times g$	centrifugal force
h	hours
HSCC	high SCC
ICSCC	individual cow SCC
IHSCC	individual heifer SCC
IMI	intra-mammary infection
IQSCC	individual quarter SCC
IRCM	incidence rate of clinical mastitis
Jun	June
kg	kilograms
LF	left fore
LH	left hind
M1	marker
MAb	monoclonal antibody
Mar	March
MBP	modified Baird Parker
MBS	MultiBlock system
MEGA	Molecular evolutionary genetics analysis
min	minutes

µl	microlitre
µm	micrometre
ml	millilitre
MLEE	multilocus enzyme electrophoresis
MLST	multilocus sequence typing
mM	millimolar
mm	millimetre
MRSA	methicillin resistant *Staphylococcus aureus*
MS	microsoft
MSSA	methicillin sensitive *Staphylococcus aureus*
n	number
NCIMB	National collection of industrial and marine bacteria
NCTC	National collection of type cultures
nm	nanometres
NMC	National mastitis council
NMR	National milk records
OD	optical density
ORF	open reading frame
PBS	phosphate buffered saline
PCA	plate count agar
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
POP6	performance optimised polymer 6
®	registered
R1/R2	regions of interest
RAPD	random amplified polymorphic DNA
RF	right fore
RH	right hind
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
S.	*Staphylococcus*
s	seconds
SBA	sheep's blood agar

SCC	somatic cell count
SE	staphylococcal enterotoxin
Sept	September
SLV	single locus variant
SSC	side scatter
ST	sequence type
START	sequence type analysis and recombinational tests
Str.	*Streptococcus*
spp.	species
TBC	total bacterial count
TCT	tube coagulase test
™	trademark
Tris	Tris hydroxymethylaminoethane
TS	teat skin
TSB	tryptone soya broth
TSST	toxic shock syndrome toxin
TVC	total viable count
UF	ultrafiltration
UK	United Kingdom
USA	United States of America
UV	ultra violet
VLA	Veterinary laboratories agency
v/v	volume:volume ratio
WinMDI	Windows multiple document interface for flow cytometry
w/v	weight:volume ratio

Man can learn nothing, except by going from the known to the unknown

Claude Bernard

Chapter 1: General introduction

1.1 Research Aims

Mastitis is one of the most common production diseases affecting dairy cattle in the UK (Esslemont and Kossaibati, 1996) and worldwide (Wells *et al.*, 1998). The traditional view of *Staphylococcus aureus* mastitis is that it is spread from cow to cow (Davidson, 1961; Newbould, 1968), and whilst it can be controlled through appropriate antibiotic use and management (Zecconi *et al.*, 2003), it remains a major cause of bovine mastitis (Zecconi and Piccinini, 2002). Treatment of infections are most effective when therapy is given early (Milner *et al.*, 1997), however lack of routine antibiotic use on organic dairies means that *S. aureus* control is more difficult than on conventional farms. There is also evidence that infected cows may not be the only reservoir of infection (Matos *et al.*, 1991; Roberson *et al.*, 1994*b*).

The main objectives of this work were therefore twofold:

- Develop and assess the usefulness of an improved method for rapid detection of *S. aureus* in milk.
- Investigate the feasibility of using multilocus sequence typing (MLST) to assess and improve understanding of the population biology and epidemiology of *S. aureus* on dairy farms.

More specifically the aims were:

i. Extract *S. aureus* from infected milk samples, and present them in a solution suitable for labelling/marking and analysis using optical instruments.
ii. Perform a longitudinal study of individual quarter and whole animal somatic cell count (SCC), and associated pathogens (where present) of organically reared heifers entering an organic herd.
iii. Carry out a longitudinal study of mammary infections in the cows recruited for *S. aureus* collection.
iv. Isolate *S. aureus* from the milk and environment of an organic dairy farm.
v. Investigate the suitability of MLST as a typing technique to study *S. aureus* on a single farm.

vi. Use MLST to investigate *S. aureus* from a number of geographical locations and environments associated with dairy cattle.
vii. Compare MLST as a method of typing *S. aureus* of bovine origin to methods used previously.
viii. Use MLST to investigate the evolutionary and population biology of bovine *S. aureus*.

This introduction focuses mainly on mastitis caused by *S. aureus*, but other pathogens are discussed where such information is pertinent.

1.2 Mastitis in dairy cattle

Bovine mastitis is defined as inflammation of the mammary gland (Kehrli and Shuster, 1994; Harmon, 1994), usually caused by intra-mammary infection (IMI) with bacteria. The inflammation reduces both milk production and quality, and consequently control is important for producers and consumers (Enevoldsen *et al.*, 1995).

Intra-mammary infections may lead to clinical or sub-clinical disease. Clinical mastitis is present when the cow has visually abnormal milk (e.g. clots, flakes or watery), which may or may not be accompanied by signs of inflammation (e.g. heat or swelling). Clinical mastitis can be divided into three presentations: mild, where only the milk is affected, and is usually treated directly by the herdsman with most of the losses due to lost milk production. Moderate cases affect milk and cause changes to the udder, and increased milk yield losses can lead to increased chances of culling. Severe mastitis infections can lead to sickness of the cow and ultimately be fatal.

Sub-clinical infections produce no obvious signs of disease (Leigh, 1999). Sub-clinical infections are usually detected by an increase in the SCC of milk, these are part of the cows natural defence mechanism and include lymphocytes, macrophages, polymorphonuclear and epithelial cells (Sordillo *et al.*, 1997). In the healthy gland SCC are often <80,000 cells ml^{-1} of milk (Laevens *et al.*, 1997). Following IMI, cells migrate to the mammary gland and a threshold of 250,000 cells ml^{-1} has been suggested as a reasonable level to detect sub-clinical infections (Dohoo and Meek, 1982). After infection has been cleared, SCC falls to a level similar to the pre-

infection concentration, although this varies for individual pathogens (de Haas *et al.*, 2002). If bacteria are able to survive the initial response, then infection continues and SCC may fluctuate but remain abnormally high even after bacteria are eliminated, until the gland heals (Harmon, 1994). Prolonged inflammation can damage mammary tissue and reduce milk yield (Milner *et al.*, 1997; Sordillo *et al.*, 1997).

In the UK, there has been a dramatic change in the incidence and aetiology of mastitis since the late 1960s. The rate of clinical mastitis has fallen and the environmental *Escherichia coli* and *Streptococcus uberis* have replaced *S. aureus* as the main causative pathogens (Booth, 1997). A major factor leading to these changes was the introduction of a plan to control udder disease (Neave *et al.*, 1966). The change in aetiology may have occurred because this method controlled contagious, but not environmental, pathogens. The principles were based on elimination of established, and reduction of new, infections. The main points were maintenance of hygiene at milking, ensuring correct milking machine function, use of post-milking teat disinfection, treatment and/or culling of clinical and recurrent cases and routine treatment of all quarters at the end of lactation with a long acting intra-mammary antibiotic (dry cow therapy; DCT). Since publication in the 1960s this method has become known as the 'Five Point Plan' and remains the recommended standard for control of mastitis.

1.3 Mastitis in organic dairy herds

Mastitis is the most frequently occurring health problem in organic dairy herds (Weller and Bowling, 2000), with *S. aureus* and *Str. uberis* (Vaarst and Enevoldsen, 1997; Busato *et al.*, 2000; Weller and Bowling, 2000; Barlow, 2001; Turner, 2001) the most frequently isolated pathogens.

Treatment and control of mastitis on organic dairies differs from conventional farms mainly in the approach to DCT, since routine use of DCT is forbidden in organic regulations (Hovi and Roderick, 1998). Conventional antibiotic therapy to treat clinical cases is also discouraged by prolonged milk withdrawal periods (Hovi and Roderick, 1998). This is reflected by the majority of cases on organic dairies initially receiving homeopathy or other non-antibiotic therapy (Hovi and Roderick,

1998; Weller and Bowling, 2000; Turner, 2001) such as ginseng (Hu *et al.*, 2001), the herb *Persicaria senegalense* (Abaineh and Sintayehu, 2001), *Calcarea fluorica* and *Hepar sulphuris*. These are administered in addition to any udder stripping and massage that may be practised (Turner, 2001).

1.4 Mastitis pathogens: duration of infection and impact on somatic cell count

Staphylococcus aureus: is characterised by an erratic shedding pattern of bacteria during infection due in part to the internalisation of bacteria and the shedding cycle (high or low) of the cow (Sears *et al.*, 1990; Buelow *et al.*, 1996*a*). Poor response to therapy leads to the persistence of *S. aureus* infections in the mammary gland (Larsen *et al.*, 2000*a*) often accompanied by increased SCC (Shoshani *et al.*, 2000; de Haas *et al.*, 2002; Middleton *et al.*, 2002). The rate of recurrent *S. aureus* mastitis i.e. two or more clinical cases caused by the same organism in the same quarter in one lactation has been reported to be more than double that of *E. coli* (Lam *et al.*, 1996).

Escherichia coli: is the most frequently isolated pathogen from cases of clinical mastitis in low SCC herds (Erskine *et al.*, 1988; Barkema *et al.*, 1998). In general infections are short-lived (Smith *et al.*, 1985), and SCC fall rapidly after infection (de Haas *et al.*, 2002). Recurrent clinical episodes of mastitis caused by *E. coli* have been demonstrated to be caused by the same strain (Lipman *et al.*, 1995; Döpfer *et al.*, 1999; Bradley and Green, 2001), implying that the bacteria survive in the host.

Streptococcus uberis: has been estimated to be responsible for up to 33% of clinical mastitis cases in the UK (Hillerton *et al.*, 1993). High levels of infection can persist in excess of 100 days (Smith *et al.*, 1985; Zadoks *et al.*, 2003), and are generally caused by the same strain, but recurrent cases can be caused by different strains (Phuektes *et al.*, 2001*b*; Wieliczko *et al.*, 2002; Zadoks *et al.*, 2003). In addition, *Str. uberis* is the most common cause of IMI in the dry cow (Todhunter *et al.*, 1995), with an increase in prevalence towards the end of the dry period (Smith *et al.*, 1985; Green *et al.*, 2002).

Coagulase-negative staphylococci (CNS): infections tend to be milder than infections with *S. aureus*, result in an increased SCC, reduced milk yield (Timms and Schultz,

1987) and can persist throughout lactation (Rainard and Poutrel, 1982). There is conflicting evidence concerning the protective effect of CNS against infections with other pathogens (Matthews *et al.*, 1991; Nickerson and Boddie, 1994; Lam *et al.*, 1997) but any protective effect has been hypothesised to be caused by competitive growth or the increased SCC (Matthews *et al.*, 1991; Lam *et al.*, 1997).

Corynebacterium bovis: is generally regarded as a minor pathogen and has been demonstrated to offer protection against infection with *S. aureus* (Brooks and Barnum, 1984*a*), although the effect varies for other pathogens (Pankey *et al.*, 1985; Hogan *et al.*, 1988; Lam *et al.*, 1997). Recently the timing of infection has been hypothesised to be important, with *C. bovis* isolation at drying off leading to an increased risk of clinical mastitis, but it's presence in late dry period/post-calving samples associated with a reduced risk of clinical mastitis (Green *et al.*, 2002), and this may be the reason for conflicting reports. There is also some evidence relating outbreaks of clinical mastitis to *C. bovis* infections (Counter, 1981; Boyer, 1998; Robinson and Harwood, 1998).

1.5 Characterisation of mastitis pathogens

Mastitis-causing bacteria have been characterised by various aspects of their population biology, though all schemes have their limitations.

Pathogenicity: Pathogens have been classed as major and minor (Rainard and Poutrel, 1982), dependent upon the level of disease manifestation they cause. Mastitis caused by major pathogens generally results in more severe disease and greater compositional change in milk. The most common major pathogens are *S. aureus*, *Str. agalactiae*, *Str. dysgalactiae*, *Str. uberis* and coliforms especially *E. coli*. Coagulase-negative staphylococci (CNS) and *Corynebacterium bovis* are classed as minor pathogens, since they are rarely associated with clinical disease (Harmon, 1994).

Reservoir: Mastitis pathogens have also been classified as environmental or contagious depending on their main route of transmission. Environmental pathogens' primary reservoir is the environment in which the cow lives and not infected mammary quarters e.g. streptococci other than *Str. agalactiae* and coliform

bacteria (Smith *et al.*, 1985). Whereas the primary reservoir of contagious pathogens is considered to be the infected quarter to which teats are exposed during milking e.g. *S. aureus* and *Str. agalactiae* (Smith *et al.*, 1985).

Research has demonstrated that these classification schemes are not definitive. *S. aureus* for example has been reported as 'ubiquitous in the dairy cow environment' (Matos *et al.*, 1991; Roberson *et al.*, 1994*b*), and associated with low SCC mastitis (Hoblet *et al.*, 1988; Torgerson *et al.*, 1992). In addition, the contagious spread and persistence of *E. coli* and *Str. uberis* have been demonstrated (Lipman *et al.*, 1995; Döpfer *et al.*, 1999; Bradley and Green, 2001; Phuektes *et al.*, 2001*b*; Khan *et al.*, 2003).

These differences make classification more difficult. In an attempt to overcome some of these problems Zadoks (2002) suggested using a sliding scale to demonstrate the epidemiology of mastitis pathogens (Fig. 1.1) where the balance changes gradually opposed to a definite split. This still simplifies the problem. For each pathogen there is likely to be a bimodal strain distribution based on epidemiology or pathogenicity (Fig. 1.2). As strain typing is increasing, it is reasonable to assume that certain aspects of this may inform further on pathogen classification systems in the future.

Investigation of virulence factors such as toxic shock syndrome toxin (TSST), staphylococcal enterotoxins (SEs) or the *coa* gene (coding for coagulase) in *S. aureus*, or the basic genetic make-up, allows strain differentiation (Schwarzkopf and Karch, 1994; Kapur *et al.*, 1995; Fitzgerald *et al.*, 1997, 2000; Stephan *et al.*, 2001). Assessment of virulence factors can be related to pathogenicity (Matsunaga *et al.*, 1993; Piccinini and Zecconi, 2001), and this may help to form part of a more suitable method of classification. Knowledge of the underlying genetic make-up would potentially provide information on global relationships and may be a sound basis for classification. Whilst this may prove valuable for contagious pathogens, it may be less so for environmental bacteria. The epidemiology of mastitis causing bacteria may be more difficult to relate to genetic variation, as it is likely to be influenced by management techniques which may vary from farm to farm.

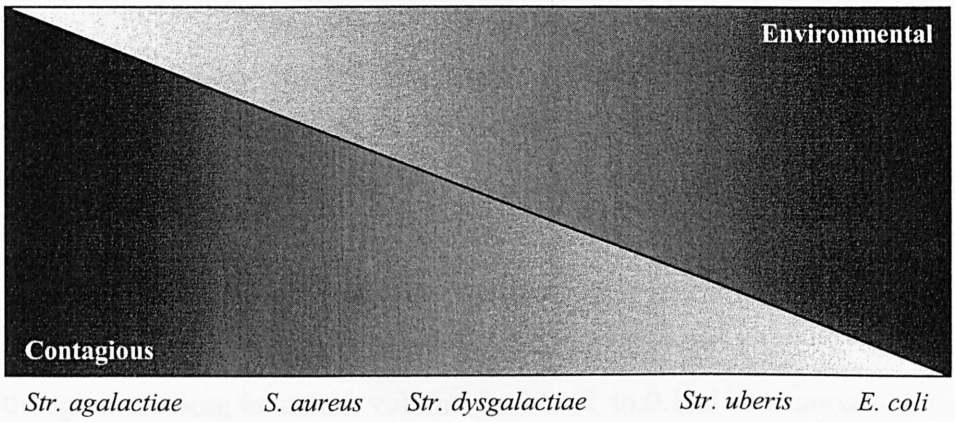


Fig.1.1. Sliding scale from contagious to environmental transmission pathways of mastitis pathogens. (Adapted from Zadoks, 2002).

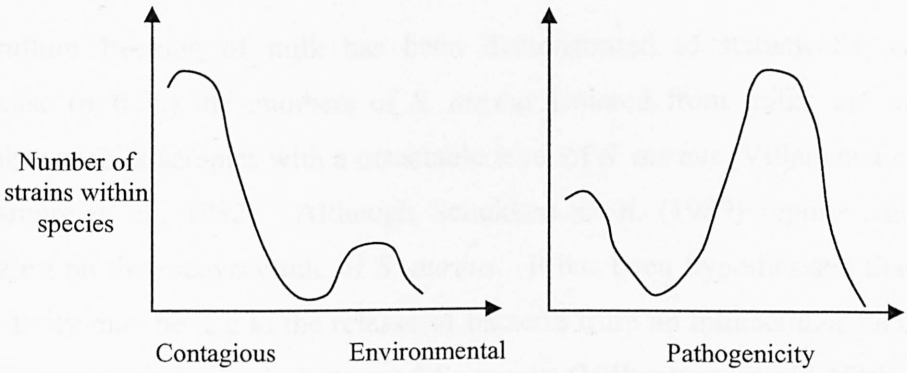


Fig. 1.2. Examples of possible bimodal strain distributions of *S. aureus* based on primary reservoir or pathogenicity.

1.6 Methods of identifying mastitis pathogens

As the need for microbiological testing in the dairy industry has increased, limitations of conventional methods have been highlighted and revealed (Vasavada, 1993). Pathogen identification assists antibiotic choice and may indicate the route of transmission (Sears and Heider, 1981; Holm and Jespersen, 2003).

Culture techniques: The National Mastitis Council (Hogan *et al.*, 1999) recommends using an inoculum of 0.01 ml for the routine culturing of quarter and composite milk samples, and greater volumes [0.1ml] for acute clinical cases.

The detection of *S. aureus* can be problematic when using culture techniques due to it's erratic shedding pattern, the dilution of infected samples by milk from uninfected

quarters and failure to inoculate an adequate volume onto culture media (Sears *et al.*, 1990; Buelow *et al.*, 1996*b*). Shedding patterns cannot be altered, although use of individual quarter milk samples and larger volumes of inoculum can improve detection sensitivity (Buelow *et al.*, 1996*a,b*). Sears *et al.* (1990) reported that experimentally the mean sensitivity of a single sample to detect *S. aureus* using an inoculum of 0.1 ml was 74.5%. This increased to 94% and 98% by use of a second and third consecutive sample respectively (taken at separate milkings). The mean sensitivity of detecting naturally occurring infections from a 0.05ml aliquot was 89%, though increasing inoculum volume from 0.01 to 0.1ml substantially increased detection sensitivity to 84-95% for detection of *S. aureus* in individual quarters (Buelow *et al.*, 1996*a,b*).

Preculture freezing of milk has been demonstrated to statistically significantly increase ($p<0.05$) the numbers of *S. aureus* isolated from milk, and increase the number of milk samples with a detectable level of *S. aureus* (Villanueva *et al.*, 1991; Dinsmore *et al.*, 1992). Although Schukken *et al.* (1989) reported no effect of freezing on the recovery rate of *S. aureus*. It has been hypothesised that increased sensitivity may be due to the release of bacteria from an intracellular location or the disruption of chains and clusters of *S. aureus* (Villanueva *et al.*, 1991; Sol *et al.*, 2002). A reduced sensitivity has been detected in post-milking compared to pre-milking samples (Sears *et al.*, 1991), although pre-culture freezing reversed these effects (Godden *et al.*, 2002). Milk sample contamination is minimised by hand delivery of samples within one day of collection (Dinsmore *et al.*, 1990).

Polymerase chain reaction (PCR): PCR methods have been developed to detect all of the main mastitis pathogens from quarter milk samples based on species-specific DNA sequences. The techniques were developed as uniplex (single species detection) (Khan *et al.*, 1998; Kim *et al.*, 2001; Martinez *et al.*, 2001; Riffon *et al.*, 2001; Meiri-Bendek *et al.*, 2002) and multiplex (multiple species detection) (Phuektes *et al.*, 2001*a*) reactions. A multiplex PCR has also been developed to screen bulk milk samples for *S. aureus*, *Str. agalactiae*, *Str. dysgalactiae* and *Str. uberis* (Phuektes *et al.*, 2003). Detection limits vary, with multiplex PCR having a 10- to 100-fold lower sensitivity than uniplex PCR (Phuektes *et al.*, 2001*a*). Without an enrichment step, uniplex PCR detected as few as 10^2 to 10^4 cfu ml^{-1}, following

enrichment, this was reduced to one cfu ml^{-1} (Martinez *et al.*, 2001; Meiri-Bendek *et al.*, 2002). While these levels of detection are impressive and in some cases supersede the 'gold standard' of culture, a positive result provides no indication of the total numbers of bacteria present in the initial sample, nor do they determine viability (Martinez *et al.*, 2001).

Alternative methods: A cow-side test to determine the Gram-staining potential of bacteria in milk has been developed (Yazdankhah *et al.*, 2001), this can provide on-site guidance for antibiotic usage, but gives no indication of level of infection and mixed infections may yield ambiguous results. A simple tube coagulase test (TCT) for detection of *S. aureus* has also been developed (Yazdankhah and Olsen, 1998), and is probably of most use to veterinary practitioners with limited facilities or dairy farmers as a simple diagnostic test, although overnight incubation may be required.

Flow cytometry (FCM): Methods have been developed to permit FCM detection of total bacteria and Gram-positive and -negative bacteria in milk (Gunasekera *et al.*, 2000; Holm and Jespersen, 2003). Other FCM analyses of milk have detected organisms associated with food spoilage and risks to public health such as *Salmonella typhimurium* (McClelland and Pinder, 1994) and *Listeria monocytogenes* (Donnelly and Baigent, 1986; Donnelly *et al.*, 1988). However, FCM sensitivity achieved thus far is 10^4 bacteria ml^{-1} milk (Gunasekera *et al.*, 2000), and the sensitivity required for individual milk samples is much lower than this e.g. Buelow *et al.* (1996*b*) used a detection limit of 20 cfu ml^{-1} for predicting IMI.

The detection of antibodies to *S. aureus* instead of the organism (D'Apice *et al.*, 1996; Iannelli *et al.*, 1998) suggested that in 25% of cases, infection could be diagnosed earlier than by culture (Iannelli *et al.*, 1998). However, antibody detection could lead to misleading results, as after the clearance of an infection there is likely to be a lag period as the antibody level declines, potentially yielding a false-positive result (Emanuelson *et al.*, 1987).

If sensitivity can be improved, FCM presents the opportunity to enumerate and determine the viability of bacteria in milk through the use of staining/labelling protocols.

Whilst all of these techniques are able to diagnose IMI to a varying extent more rapidly than culture, they do not permit analysis below the species level. In order to assess variation at this level pathogens have to be isolated from milk.

1.7 Treatment of mastitis

Treatment with intramammary antibiotic therapy is limited in its efficacy, perhaps achieving only a 60% bacteriological cure for streptococcal mastitis and rarely >30% for mastitis caused by *S. aureus* (Hillerton *et al.*, 1995). Poor response of *S. aureus* to antibiotic therapy is due, in part, to antibiotic resistance (Østerås *et al.*, 1999) and the ability of *S. aureus* to resist phagocytosis and remain viable within macrophages (Craven and Anderson, 1984; Barrio *et al.*, 2000; Hébert *et al.*, 2000). A possible mechanism conferring resistance is the production of a slime capsule (Barrio *et al.*, 2000).

However, experimentally, it has been shown that early treatment of mastitis can lead to high rates of clinical and bacteriological cure being achieved more rapidly than conventional treatment. This prevents the onset of clinical signs and results in a statistically significantly shorter convalescence of the cow (Milner *et al.*, 1997; Hillerton and Semmens, 1999). In turn less milk is wasted, there are shorter milk withdrawal periods and reduced amounts of antibiotics and labour are used (Milner *et al.*, 1997). In practice these levels are difficult to achieve, as detection is usually based on clinical signs or possibly monthly SCC reports. Once detected, treatment is usually initiated without pathogen speciation and this can lead to sub-optimal antibiotic use. The development of a rapid test permitting speciation of the organisms in milk before the presence of clinical signs would facilitate earlier, and potentially more effective, treatment.

1.8 Risk factors for *Staphylococcus aureus* mastitis

As mastitis is an infectious disease, infected cows are the main source, but there are other potential risk factors. Management factors include the source of drinking water (private source increased the risk) for low BMSCC (<150,000 cells ml^{-1}) herds (Schukken *et al.*, 1991), and checking the first few streams of milk (Schukken *et al.*, 1991; Elbers *et al.*, 1998; Barkema *et al.*, 1999; Peeler *et al.*, 2000). Identification of

foremilking as a risk factor may be associated with increased levels of exposure to *S. aureus*, and suggests the importance of the possible spread of contagious pathogens at milking (Schukken *et al.*, 1991). Though it is questionable whether this finding is due to increased risk or improved detection (Elbers *et al.*, 1998; Peeler *et al.*, 2000), as is also the case for the visual checking of dry cows (Barkema *et al.*, 1999).

Barkema *et al.* (1998, 1999) reported that a BMSCC of 250-400,000 cells ml^{-1} was a risk factor for *S. aureus* mastitis, although this is probably an effect of infection (Barkema *et al.*, 1999). Conversely, Elbers *et al.* (1998) reported that a lower BMSCC (<150,000 cells ml^{-1}) was associated with increased rates of *S. aureus* mastitis. This may be because farmers better at diagnosing mastitis diverted more high SCC milk away from the bulk tank, leading to high rates of clinical mastitis but lower BMSCC (Elbers *et al.*, 1998). There may also be variation in the true rate of mastitis and that reported by farmers (Elbers *et al.*, 1998; Peeler *et al.*, 2000). Record keeping (or not), skill of detection and farmer motivation may all have an effect on the level of unreported cases, all of which will affect the incidence rate of clinical mastitis (IRCM) (Elbers *et al.*, 1998; Peeler *et al.*, 2000).

There is a general agreement that the risk of *S. aureus* IMI increases with parity on organic and conventional dairies (Shpigel *et al.*, 1998; Busato *et al.*, 2000; Zadoks *et al.*, 2001). Although stage of lactation effects are less clear as early and late lactation have been reported as periods of increased risk (Vaarst and Enevoldsen, 1997; Elbers *et al.*, 1998; Shpigel *et al.*, 1998; Busato *et al.*, 2000). These differences are probably due to variation in the classification of early, mid and late lactation in the individual reports, and greater homology between investigations would allow for more definitive conclusions to be drawn.

Quarter position may affect rates of mastitis and IMI, although this varies between studies. In general, rear quarters are considered to be at greater risk than fore quarters (Barkema *et al.*, 1997; Shpigel *et al.*, 1998; Busato *et al.*, 2000). Although Zadoks *et al.* (2001) reported that the right side of the udder was at greatest risk, and Barkema *et al.* (1997) determined that the right fore quarter was at increased risk compared to the left fore quarter. These variations may be influenced by lying

position (Ewbank, 1966) or pathogen specific effects such as transmission pattern (Barkema *et al.*, 1997).

1.9 Molecular epidemiology

The ability to accurately identify strains of infectious agents that cause disease is central to epidemiological surveillance (Maiden *et al.*, 1998) and observational studies. Molecular refers to techniques that characterise amino, or nucleic acid content, and epidemiology is the study of the incidence and distribution of diseases in populations (Foxman and Riley, 2001). DNA-based techniques are one of the most effective methods of grouping outbreak related strains (Tenover *et al.*, 1994), have high levels of resolution (Struelens *et al.*, 1996*a*) and require very little starting material. However molecular methods currently in use have significant drawbacks including inadequate discrimination and poor reproducability within and between laboratories. This latter limitation is potentially the most important of current typing methods, particularly at the inter-laboratory level (Maiden *et al.*, 1998).

Molecular typing techniques address two types of epidemiological question: one, whether isolates obtained from a localised disease outbreak are the same strain (short-term or local epidemiology); and two, how are disease causing strains from one geographical area related to those isolated from another area? (long-term or global epidemiology) (Maiden *et al.*, 1998; Spratt, 1999; Enright and Spratt, 1999).

Molecular typing methods can be categorised as single or multilocus techniques (Spratt, 1999), and both can give high levels of discrimination (Maiden *et al.*, 1998). Single locus investigations are based on a highly variable site (e.g. the *coa* gene; Schwarzkopf and Karch, 1994; Raimundo *et al.*, 1999) so that randomly selected isolates will possess distinguishable forms (or alleles) (Spratt, 1999). For bacterial pathogens, population methods based on these principles are pulsed-field gel electrophoresis (PFGE) and arbitrarily primed PCR (AP-PCR) (Maiden *et al.*, 1998; Enright and Spratt, 1999). However, the indexed variation is evolving very rapidly, usually for unknown reasons and so these methods are best suited to short-term epidemiological investigations (Maiden *et al.*, 1998; Spratt, 1999; Enright and Spratt, 1999). The rapid accumulation of variation is disadvantageous for investigations of long-term or global epidemiology (Enright and Spratt, 1999). Genes which

diversify slowly by the random accumulation of neutral or nearly neutral variation (e.g. housekeeping genes) should provide more reliable information on relationships between isolates (Maiden *et al.*, 1998; Spratt, 1999). However, these genes are relatively uniform in sequence and single locus analysis is not discriminatory enough, therefore a multilocus approach is used (Spratt, 1999) which enables a vast number of genotypes to be distinguished (Enright and Spratt, 1999) e.g. multilocus enzyme electrophoresis (MLEE) and multilocus sequence typing (MLST).

Pulsed-field gel electrophoresis (PFGE): PFGE involves digesting chromosomal DNA of organisms with restriction endonucleases (typically *Sma*I for *S. aureus*; Tenover *et al.*, 1995). The restriction fragments are resolved into a pattern of discrete bands in an agarose gel and compared to determine their relatedness (Tenover *et al.*, 1995; Olive and Bean, 1999). Tenover *et al.* (1995) suggested guidelines for the interpretation of PFGE patterns: 2-3 fragment differences indicate closely related isolates, 4-6 differences possibly related isolates, and more than 7 differences indicate unrelated isolates.

Multilocus enzyme electrophoresis (MLEE): MLEE analyses the electrophoretic mobilities of housekeeping enzymes on starch gels and equates different charge variants of each enzyme with alleles at each locus. Although each locus only generates a small number of variants, high levels of resolution are achieved by analysing a number of loci e.g. 13, with the alleles at each locus defining an electrophoretic type (ET) (Kapur *et al.*, 1995; Fitzgerald *et al.*, 1997).

Multilocus sequence typing (MLST): MLST is based on the principles of MLEE but assigns alleles at each locus based on nucleotide sequence (Enright and Spratt, 1999). This reveals all of the variation and therefore the number of alleles detected at each locus is much higher than for MLEE (Enright and Spratt, 1999). The sequences of 450-500bp internal fragments of seven housekeeping genes are determined for each isolate. This length of DNA fragment can be accurately sequenced on both strands using a single pair of primers, and for most species, provides sufficient variation to recognise many different alleles (Spratt, 1999; Urwin and Maiden, 2003).

For each gene, the different sequences are assigned as individual alleles, and the alleles at the seven loci provide an allelic profile (AP), which unambiguously defines the sequence type (ST) of the isolate (Enright and Spratt, 1999; Urwin and Maiden, 2003). No weighting is given to take into account the number of nucleotide differences between alleles, as it is not possible to determine whether differences are due to multiple point mutations or single recombinational events (Spratt, 1999; Enright and Spratt, 1999). The large number of alleles at each locus provides the ability to distinguish billions of STs, and it is extremely unlikely that two unrelated strains would have the same ST (Spratt, 1999). The ST of an isolate is sufficiently stable over time for the method to be ideal for global epidemiology (Enright and Spratt, 1999).

The relatedness of the isolates can be displayed in a number of ways including dendrograms or diagrams constructed using the BURST (Based Upon Related Sequence Types) and eBURST (enhanced BURST) algorithms (Enright *et al.*, 2002; Feil *et al.*, 2004). Dendrograms can be constructed based on allelic profiles or concatenated sequences of the seven gene fragments (Enright and Spratt, 1999). BURST diagrams divide the isolates into clonal complexes (CC) based on allelic profiles, with no isolate differing at more than three loci from any other in the CC (Enright *et al.*, 2002; Urwin and Maiden, 2003). Differences at more than three of seven loci are likely to be unreliable and should not be taken to infer phylogeny (Spratt and Maiden, 1999; Spratt, 1999). Putative ancestral strains are identified and placed in the centre of the diagram. Isolates which differ at a single locus (single locus variants; SLVs) are placed in a circle around the ancestral strain, double locus variants (DLVs) are placed in a circle around the SLVs (Enright *et al.*, 2002). A hypothetical BURST diagram is presented in Fig. 1.3. CCs are named on the basis of the ancestral strain, e.g. Fig. 1.3 displays CC1.

A major advantage of MLST is the electronic portability of DNA sequences. This allows any laboratory to characterise isolates by submitting the sequences of the seven gene fragments via the internet to a central MLST website (http://www.mlst.net).

ST	Allelic profile							
1	1	1	1	1	1	1	1	Anc.
2	1	1	1	1	1	1	2	
3	1	2	1	1	1	1	1	SLV
4	1	1	1	2	1	1	1	
5	2	1	2	1	1	1	1	
6	1	2	1	2	1	1	1	DLV
7	2	1	1	1	1	1	2	

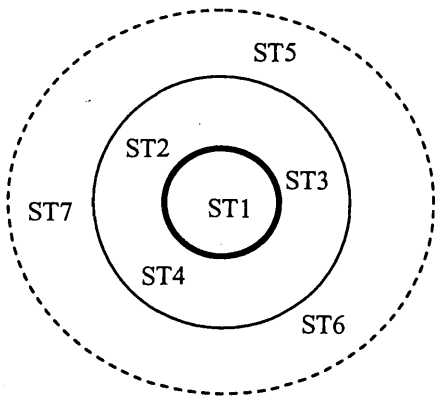


Fig. 1.3. Example BURST diagram, with sequence type and allelic profile information.
ST=sequence type, Anc.=putative ancestral strain, SLV=single locus variant, DLV=double locus variant.

MLST was first described and evaluated by Maiden *et al.* (1998) who applied the technique to a previously characterised collection of *Neisseria meningitidis*, although only six loci were used. MLST was able to resolve the major meningococcal lineages responsible for invasive disease worldwide and produced a dendrogram which was highly congruent with that obtained from MLEE data. A modified scheme using seven loci is now used, which has improved the level of discrimination and resolution between the major invasive lineages (Spratt, 1999; Enright and Spratt, 1999). Meningococcal clones diversify relatively rapidly, predominantly by recombinational exchanges with segments from other lineages (Maiden *et al.*, 1998; Enright and Spratt, 1999; Spratt and Maiden, 1999). The ability of MLST to identify these unstable clones indicates that it should provide a reliable method to identify clones within other bacterial species subject to lower rates of clonal division (Enright and Spratt, 1999; Spratt and Maiden, 1999).

Since 1998, MLST schemes have been developed for *Str. pneumoniae* (Enright and Spratt, 1998), *S. aureus* (Enright *et al.*, 2000), *Campylobacter jejuni* (Dingle *et al.*, 2001), *Str. pyogenes* (Enright *et al.*, 2001), *Haemophilus influenzae* (Meats *et al.*, 2003), *Burkholderia pseudomallei* and *Burkholderia mallei* (Godoy *et al.*, 2003), *Candida albicans* (Bougnoux *et al.*, 2003), *Enterococcus faecium* (Homan *et al.*, 2002), *Str. suis* (King *et al.*, 2002), *Str. agalactiae* (Jones *et al.*, 2003) and *Helicobacter pylori* (http://www.mlst.net). Currently MLST schemes are under development for *Acinetobacter baumanii*, *Salmonella enterica*, *Moraxella catarrhalis*, *Bacillus* spp., *Burkholderia cepacia*, *E. coli*, *Klebsiella pneumoniae*, *L. monocytogenes* (Salcedo *et al.*, 2003), *Str. uberis*, *Bordatella pertussis*, *parapertussis*

and *bronchiseptica*, *Vibrio* spp. and *Vibrio vulnificus*, *Pasteurella multocida*, *Str. mutans* and *Pseudomonas aeruginosa*. This list of developed and developing MLST schemes is continually expanding, with additional schemes appearing regularly in the literature (Urwin and Maiden, 2003).

The MLST database for each pathogen will increase as more allelic profiles are submitted potentially along with data relating to source and clinical manifestation, creating a single internet-based resource for global epidemiology. This will allow the detection and monitoring of the spread of new strains or clones.

1.10 History of *Staphylococcus aureus*

In addition to *S. aureus* being an important mastitis pathogen, it is also an important opportunistic pathogen causing a wide variety of community and hospital acquired infections in humans (Booth *et al.*, 2001; Grundmann *et al.*, 2002*a*) including several that are life threatening (Fitzgerald and Musser, 2001). *S. aureus* is an adaptable opportunistic pathogen able to infect, persist and replicate in a number of environments including soft tissues, visceral organs and bone (Coulter *et al.*, 1998). This ability of *S. aureus* may be due to it's extensive genetic variation, as approximately 22% of genes are associated with strain-specific functions (Fitzgerald and Musser, 2001). Therefore the investigation, detection and identification of globally significant strains is important with respect to developing effective anti-staphylococcal therapies. One of the most serious contemporary challenges to successful treatment of these infections is the appearance and global spread of methicillin-resistant *S. aureus* (MRSA) (Jevons, 1961; Crisóstomo *et al.*, 2001). The initial stage in the emergence of this pathogen was the acquisition of the *mec* gene (Crisóstomo *et al.*, 2001). MRSA evolution has occurred independently with horizontal transfer of the *mec* gene from an unknown source into at least five distinct chromosomal lineages (Fitzgerald and Musser, 2001; Fitzgerald *et al.*, 2001). The recent emergence of strains with reduced susceptibility to vancomycin (Midolo *et al.*, 2003) is also a cause for concern.

1.11 Multilocus sequence typing of *Staphylococcus aureus*

MLST of human *S. aureus* isolates has been used to investigate a geographically localised population (Day *et al.*, 2002; Feil *et al.*, 2003) and characterise and study

MRSA evolution (Enright *et al.*, 2000; Crisóstomo *et al.*, 2001; Enright *et al.*, 2002; Feil *et al.*, 2003).

Studies based on a localised population divided the isolates into nine CCs, two (CC30/CC39) were closely related. There was no statistically significant difference in the distribution of STs isolated from carriers and those associated with invasive disease within CCs (Day *et al.*, 2002; Feil *et al.*, 2003). Thus based on MLST there was no evidence for the existence of hypervirulent clones (Feil *et al.*, 2003).

The examination of MRSA clones (Enright *et al.*, 2000, 2002; Crisóstomo *et al.*, 2001) demonstrated that the earliest detected MRSA isolates (Jevons, 1961) had the same or similar STs to contemporary isolates (Crisóstomo *et al.*, 2001), and that some methicillin susceptible *S. aureus* (MSSA) had identical allelic profiles to some MRSA isolates (Enright *et al.*, 2000). This is probably due to MSSA isolates that have gained the *mec* element have not yet had sufficient time to accumulate sequence variation within their housekeeping genes (Enright *et al.*, 2000). This pattern of evolution of MSSA strains acquiring the *mec* element is supported by further work of Enright *et al.* (2002) who suggested putative evolutionary pathways of a number of MRSA isolates.

1.12 Strain typing of bovine *Staphylococcus aureus*

PFGE (Zadoks *et al.*, 2000; Zschöck *et al.*, 2000; Buzzola *et al.*, 2001; Middleton *et al.*, 2002; Sommerhäuser *et al.*, 2003), MLEE (Kapur *et al.*, 1995; Fitzgerald *et al.*, 1997) and binary typing (BT) (Zadoks *et al.*, 2000) have been used to type *S. aureus* of bovine origin. There is general agreement that *S. aureus* displays clonal relationships on farms, indicated by limited diversity and the dominance of one or two strains. It has also been suggested that relatively few clones of *S. aureus* are responsible for the majority of IMI (Kapur *et al.*, 1995; Aarestrup *et al.*, 1997; Fitzgerald *et al.*, 1997; Annemüller *et al.*, 1999; Zadoks *et al.*, 2000). Although some studies have reported that no strains of *S. aureus* were common to more than one farm and indicated a sporadic pattern of infection more akin to environmental pathogens (Joo *et al.*, 2001; Sommerhäuser *et al.*, 2003). This may be evidence of a more environmental style *S. aureus* infection, as suggested by Zadoks (2002).

The variation in these findings may be because various typing techniques and methods of interpretation were used. There is also likely to be differences in management, since the data originated from Finland (Aarestrup *et al.*, 1997), Germany (Annemüller *et al.*, 1999; Sommerhäuser *et al.*, 2003), Korea (Joo *et al.*, 2001), The Netherlands (Zadoks *et al.*, 2000) and Sweden (Aarestrup *et al.*, 1997).

An environmental style pathogen suggests that sources other than the lactating mammary gland exist. Zadoks *et al.* (2000) reported that the predominant *S. aureus* genotype in the milking herd differed from isolates in heifers, and almost 25% of strains detected in heifer IMI at parturition have been reported as previously undetected on the farm (Roberson *et al.*, 1998). Heifer body sites have also been identified as the only possible source for 5% of heifer IMI (Roberson *et al.*, 1998). A Danish study (Larsen *et al.*, 2000*a*) reported that strains of *S. aureus* isolated from IMI could also be isolated from skin lesions, suggesting that they may serve as a reservoir of infection able to cause mastitis, or vice versa.

A better understanding of the population biology and epidemiology of bovine *S. aureus* is therefore required. The identification of important/widely distributed strains/clones will assist in determining targets for control measures.

Chapter 2: Development of a method allowing the rapid detection of *Staphylococcus aureus* in milk[1]

2.1 Introduction

Determination of the microbiological content of raw milk is of interest and importance to milk producers, processors and consumers. The motivation for developing rapid methods to obtain this information is obvious: identification of a problem sooner, allows the elimination process to be initiated earlier (Holm and Jespersen, 2003).

A number of techniques have been developed which allow the determination of bacterial spp. and the host immune response (somatic cell count; SCC) in milk, based on flow cytometry (FCM) (McClelland and Pinder, 1994; Donnelly and Baigent, 1986; Ianelli *et al.*, 1998; Gunasekera *et al.*, 2000; Rivas *et al.*, 2001; Holm and Jespersen, 2003; Oliveira *et al.*, 2003). Techniques based on antibody labelling of bacteria for detection by flow cytometry have been developed to detect *Listeria monocytogenes* (Donnelly and Baigent, 1986) and *Salmonella typhimurium* (McClelland and Pinder, 1994) in milk. A FCM method for distinguishing Gram-positive and Gram-negative bacteria in milk has been described (Holm and Jespersen, 2003) as has a technique for measuring the total bacterial count (TBC) of milk (Gunasekera *et al.*, 2000). Monitoring of overall mammary gland health by FCM, putatively identifying three mammary gland health related conditions (non-mastitic, early inflammatory and late inflammatory) has also been demonstrated (Rivas *et al.*, 2001). However, none of these techniques are aimed at the direct detection of any of the main bovine mastitis pathogens.

The rapid detection of *S. aureus* in milk can reduce the time taken to the initiation of treatment, leading to improved cure rates and reduced overall costs (Milner *et al.*, 1997; Hillerton and Semmens, 1999). Enumeration of the bacteria may also be important, although with respect to *S. aureus* this may be dependent on the stage of infection (Sears *et al.*, 1990).

[1] Some of the work presented in this chapter was published in *Applied and Environmental Microbiology* (2003), **69** (1), 719-721. A reprint of this publication is in Appendix 1.

The aim of the work presented in this chapter was to assess different methods of detecting *S. aureus* in milk, and to develop a protocol that allowed detection and enumeration of *S. aureus* in milk rapidly and specifically. A flow cytometer was chosen as the appropriate instrument to use with this technique.

2.2 Materials and methods

2.2.1 Flow cytometry methodology

A Becton Dickinson (BD) FACScan™ flow cytometer was used to analyse milk samples as part of this project. The instrument was calibrated using BD CaliBRITE™ three-colour beads as recommended by the manufacturer, and following expert advice (D. Mason, *personal communication*). These were used under software control to adjust instrument settings and check sensitivity.

Samples were presented to the instrument in 5ml (12×75mm) round bottom polystyrene Falcon tubes (35 2052; BD, Cowley, Oxford, UK) and a user defined amount analysed, either a length of time e.g. 10s, or analysis of a set number of 'events'. Data were presented in the form of forward scatter (FSC) by side scatter (SSC) graphs and fluorescence histograms, and were saved for later analysis using Windows Multiple Document Interface for flow cytometry (WinMDI) software (Joseph Trotter, The Scripps Institute, CA).

2.2.2 Spectroscopy methodology

A Thermo Spectronic Unicam (Thermo Electron Spectroscopy, Cambridge, UK) was used to determine optical density, the software carried out a series of instrument self-checks of the lamps and optics before setting default values at start-up.

Prior to sample analysis a baseline was set using sterile distilled water, to calibrate the instrument. Samples were interrogated in the 'sample' chamber (while sterile distilled water remained in the 'reference' chamber) at a user-defined wavelength in a 1cm light path. Both disposable (67.742; Sarstedt Ltd., Beaumont Leys, Leicester,

UK) and black-sided (104 B–OS; Hellma UK Ltd., Southend-on-Sea, Essex) cuvettes were used, and data were presented as optical density (OD) readings.

2.2.3 Phase contrast and fluorescence microscopy

All slides were visualised using an Olympus BX60 light/fluorescence microscope fitted with a 100 watt mercury arc lamp (USH-102D, Ushio) for fluorescence illumination. Slides were viewed under oil immersion using a 100× objective lens (1,000× total magnification). Kodak Ektachrome P1600× film was used for all photography in an Olympus 35mm camera controlled by an Olympus PM-20 exposure control unit. All films were developed using push processing by Colab (Coventry, UK).

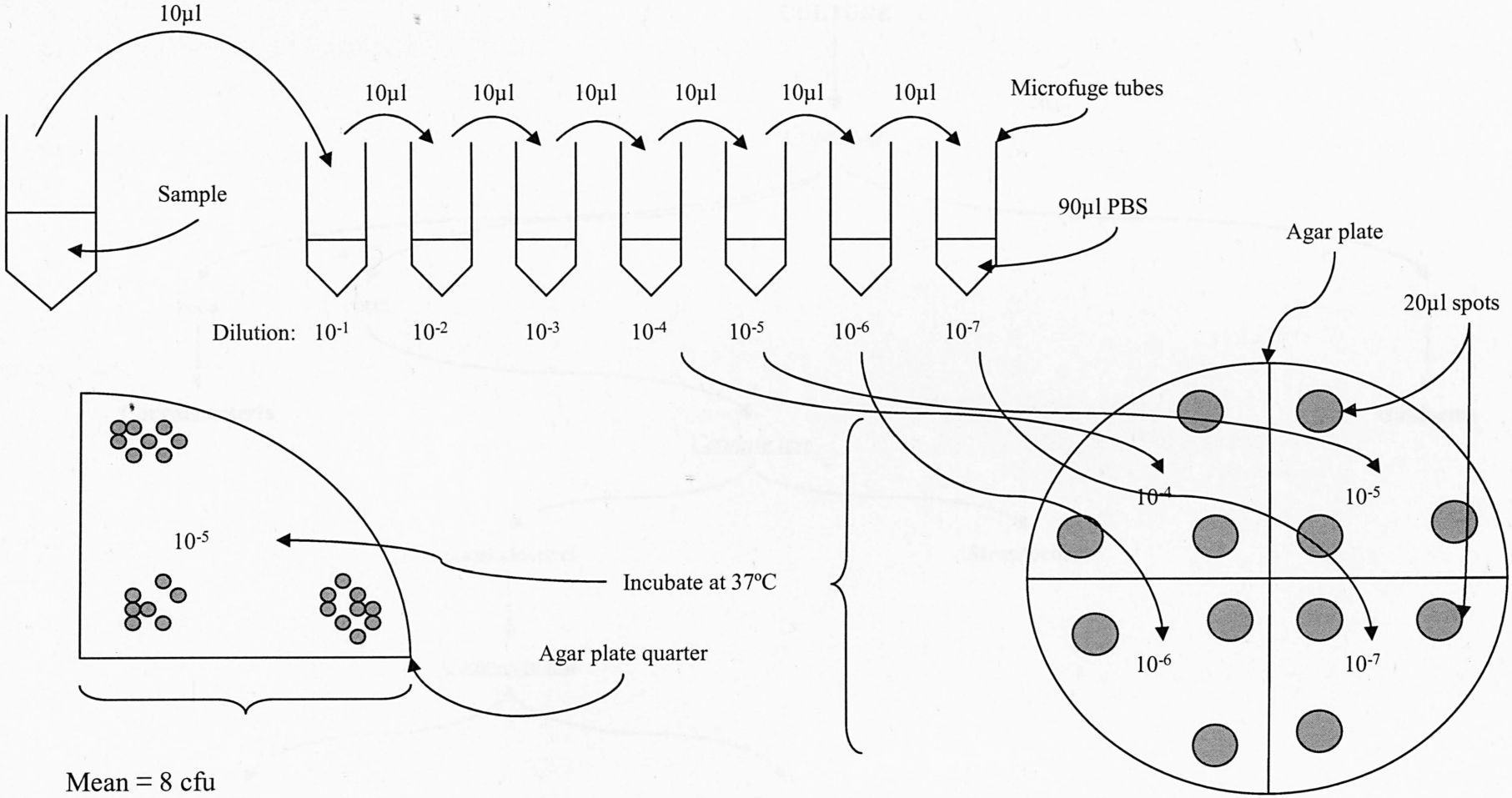
2.2.4 Plate count method for determining total viable counts

A diagrammatic representation of the method is presented in Fig. 2.1. A ten-fold dilution series with sterile phosphate buffered saline (PBS) diluent was used. Three 20µl aliquots of four dilutions were spotted onto separate quadrants of an agar plate (5% sheep's blood agar (SBA) or plate count agar (PCA)). The spots were dried and the plates incubated inverted overnight at 37°C. Colony forming units (cfu) were determined for each spot, and a TVC (expressed as cfu ml^{-1}) was calculated for the original sample.

2.2.5 Routine methods of bacterial identification

Traditional mastitis bacteriology: Raw milk samples thought to be infected were obtained from farms in Somerset (*courtesy of*: Martin Green, University of Warwick). One-hundred microlitres of each sample was spread across the surface of a 5% SBA and MacConkey agar plate, and incubated inverted at 37°C overnight.

Colony morphology was noted after 24h, Gram stains and biochemical tests as outlined in Fig. 2.2 were used to identify the cultures, as recommended by the National Mastitis Council (NMC; Hogan *et al.*, 1999). All Gram-positive, catalase-positive cultures were streaked across modified Baird Parker (MBP) agar as this is selective for *S. aureus*.



Mean = 8 cfu

= 400 cfu ml^{-1} (10^{-5} dilution)

= **4×10^7 bacteria ml^{-1} (original sample)**

Fig. 2.1. Plate count method for determining total viable counts.

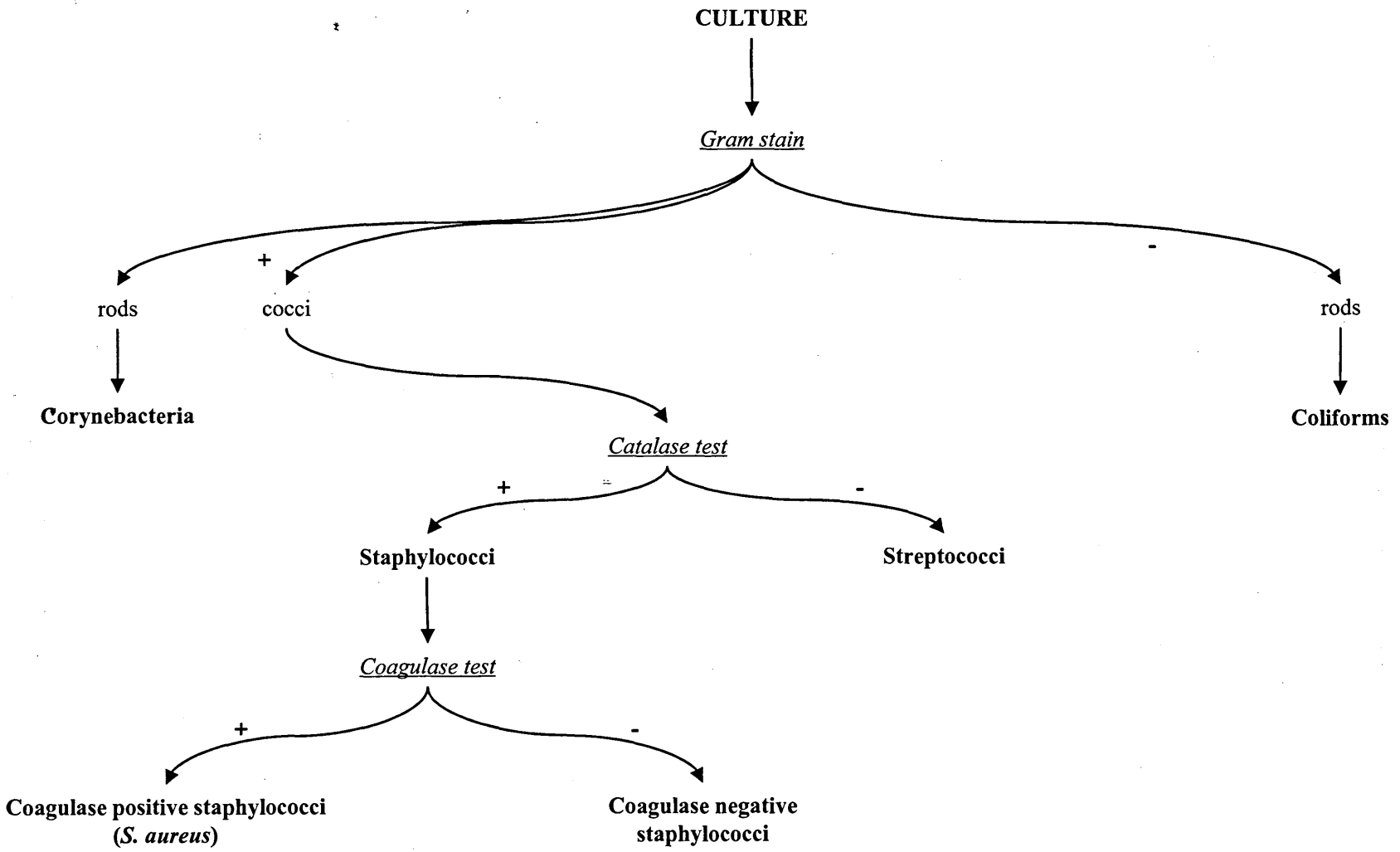


Fig. 2.2. Tests used to identify bacteria isolated from milk samples. (Adapted from NMC (Hogan *et al.*, 1999))

Use of API strips: All API strips were inoculated, incubated and interpreted using the identification software as recommended by the manufacturer (API Staph and API Strep package inserts).

2.2.6 Development of a method allowing the rapid detection of *S. aureus* in milk

The method developed by Gunasekera *et al.* (2000) for the rapid detection of total bacteria in milk by flow cytometry was identified as a potentially suitable method of milk preparation.

Milk needs to be treated with an enzyme to remove or modify proteins, as they can bind non-specifically to fluorescent stains and interfere with the staining and detection of bacteria (Gunasekera *et al.*, 2000).

Gunasekera *et al.* (2000) used 0.05mg of proteinase K (P6556; Sigma-Aldrich Co. Ltd.) or 10µl of savinase® (Novozymes, Bagsværd, Denmark) to treat 100µl of milk. Treated samples were incubated at 37°C for 30 to 45min, and 900µl of 150mM NaCl was then added and mixed by inversion. Samples were centrifuged at 14,000×*g* for 10min and the top layer (lipids and digested proteins) drawn off without disturbing the pellet which contained bacteria. The pellet was resuspended in 100µl of 150mM NaCl for analysis.

This method was adapted and refined to isolate *S. aureus* from milk and present them in a solution suitable for use with a fluorescent antibody labelling protocol. The experiments conducted to optimise the protocol, and the individual effects on bacterial recovery are summarised in Table A2.1 in Appendix 2.

2.2.7 Effect of milk clearing on bacterial recovery and milk optical density

In addition to the enzymes used by Gunasekera *et al.* (2000), a sample of alcalase was obtained (Novozymes). This is designed to break down proteins in milk in the manufacture of infant milk formula (http://www.novozymes.com). The effect of alcalase® (Novozymes), savinase® (Novozymes), and proteinase K (P6556; Sigma-Aldrich) on *S. aureus* (NCIMB 702892) and a range of *S. aureus* from clinical and sub-clinical mastitis, suspended in tryptone soya broth (TSB) and milk, were investigated. The enzymes were diluted 1:10 with the *S. aureus* suspension, samples

were taken immediately and TVC determined. Bacterial/enzyme dilutions were incubated at 37°C overnight and re-analysed after 24h. For the suspensions in milk, samples were taken after 0, 60, 120, 180 and 300min and analysed by spectroscopy (1:50 dilution) and TVC.

2.2.8 *S. aureus* antibody labelling protocol

A primary rabbit anti-*S. aureus* monoclonal antibody (MAb) (Autogen Bioclear UK Ltd.) was diluted 1:40 with *S. aureus* suspended in 0.5% bovine serum albumin (BSA; A3350; Sigma-Aldrich) and incubated at room temperature for 30–45min. The solution was centrifuged at 16,060×*g* for 2min, 90µl of supernatant removed and discarded, and the remaining 10µl resuspended in 80µl 0.5% (w/v) BSA (Sigma-Aldrich) and 10µl fluorescein isothiocyonate (FITC) conjugated secondary goat-anti-rabbit MAb. This solution was incubated for 30–45min at room temperature and centrifuged as before, 90µl of supernatant removed and discarded, and the remaining 10µl resuspended in 90µl 0.5% (w/v) BSA (Sigma-Aldrich). A 5µl aliquot of the solution was then transferred to a slide and visualised using fluorescence microscopy (Olympus BX60).

Effects of reducing the concentration of the secondary [FITC conjugated] MAb (Autogen Bioclear) were investigated by adding 1, 2 or 5µl and adjusting the BSA volume accordingly.

2.2.9 Testing the *S. aureus* antibody specificity

Test micro-organisms were provided by Dr A. J. Bradley (University of Bristol) and purchased from the Veterinary Laboratories Agency (VLA, Bury St. Edmunds, UK), to test the specificity of the *S. aureus* MAb, using the 10µl inclusion level.

2.3 Results

2.3.1 Routine methods of bacterial identification

Traditional mastitis bacteriology: Results of culture and biochemical tests are presented in Table 2.1. Of 13 milk samples, three were contaminated (3 or more distinct colony types; Hogan *et al.*, 1999) and no growth was detected in one. The remaining nine samples yielded 14 individual colony types. All 14 were Gram-positive cocci, 11 were catalase-positive and three were catalase-negative. The catalase-negative cultures were classed as streptococci (Fig. 2.2), and the catalase-positive cultures, staphylococci. Of these four were coagulase-positive and seven coagulase-negative.

API Strips: The results of the API tests are included in Table 2.1. There are 8/14 (57.1%) ambiguous identifications. As the cultures were of veterinary origin, this gave rise to alternative possible identifications, particularly with suspected *S. aureus* isolates, when often the warning was given that the isolate may have been *S. intermedius* and further tests were required. The results of the API Strep system also had a low level of discrimination, according to the identification software.

Table 2.1. Results of the biochemical tests and API analyses of 13 milk samples.

Sample ID[1]	Gram stain	Catalase	Coagulase	MBP	API kit	API results
Duchy a	+	+	+	+	Staph	*S. aureus**
Duchy b	+	+	-	+	Staph	*S. xylosus*
Dimpie	+	+	+	+	Staph	*S. aureus**
Freda	+	+	-	+	Staph	*Micrococcus spp.*
10	+	+	+	+	Staph	*S. aureus**
64 a	+	+	-	+	Staph	*S. haemolyticus*
64 b	+	+	-	+	Staph	*S. lentus**
79 a	+	-			Strep	*Lc. lactis cremoris**
79 b	+	+	-	+	Staph	*S. xylosus*
93	+	+	-	+	Staph	*S. sciuri*
98 a	+	+	+	+	Staph	*S. sciuri*
98 b	+	-			Strep	*Str. agalactiae**
124 a	+	-			Strep	*Lc. lactis cremoris**
124 b	+	+	-	+	Staph	*S. xylosus**
46	Cont.					
53	Cont.					
67	Cont.					
96	NGD					

[1]a/b after the sample name denotes different colony types isolated from the same sample, +=positive reaction, -=negative reaction, Cont.=contaminated sample, NGD=no growth detected, *S.=Staphylococcus, Lc.=Lactococcus, Str.=Streptococcus*, *=ambiguous identification.

2.3.2 Development of a method allowing the rapid detection of *S. aureus* in milk

A pure population of *S. aureus* was easily detected by FCM when suspended in 0.5% BSA (Figs. 2.3 and 2.4). This was used to determine the region of interest (R1) for FCM analysis of treated milk samples.

The method developed involved treating 1ml of milk with 100µl alcalase and incubating at 37°C for 45min. The sample was centrifuged at 10,000×*g* for 10min, the supernatant, fats and digested proteins removed and the pellet resuspended in 1ml 0.5% (w/v) BSA. The sample was then labelled and analysed using FCM (see Section 2.2.8 for the labelling protocol).

Analysis of treated milk by FCM demonstrated 'events' in the same position on light scatter graphs as *S. aureus* suspended in 0.5% (w/v) BSA (Figs. 2.5 and 2.6). The associated histograms confirmed that the majority of these events were fluorescently labelled (Fig. 2.6 and Table 2.2), indicating that they were *S. aureus*. The marker (M1) present on the fluorescence histograms indicates the detected fluorescence, anything outside this area was classed as background noise.

FCM analysis of milk also identified a second region of interest (R2; Fig. 2.7), the majority of events detected in this region were fluorescently labelled (Table 2.2) suggesting that they were *S. aureus*.

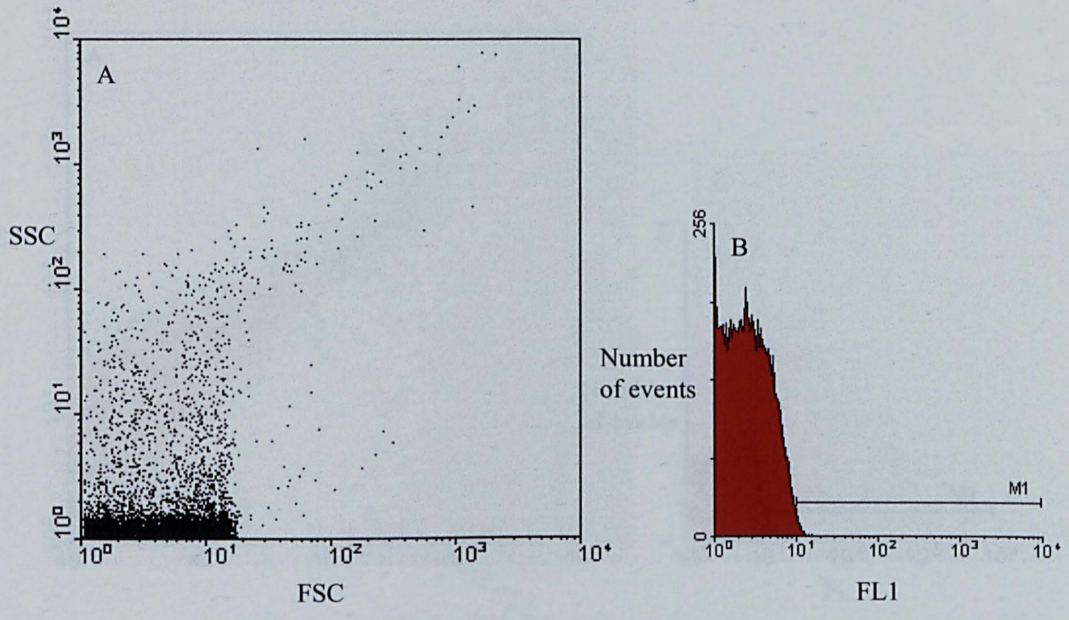


Fig. 2.3. Flow cytometry analysis of 0.5% BSA presented as (A) light scatter graph and (B) fluorescence histogram.
SSC=side scatter, FSC=forward scatter, FL1=fluorescence, M1=marker indicating area of fluorescence above background level.

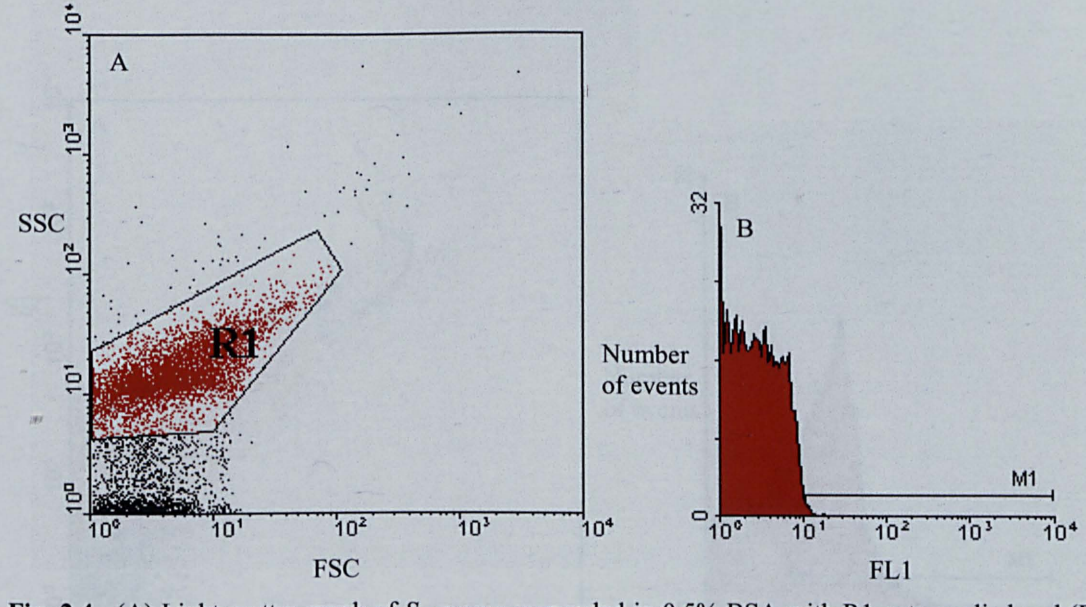


Fig. 2.4. (A) Light scatter graph of *S. aureus* suspended in 0.5% BSA with R1 gate applied and (B) fluorescence histogram of R1.
SSC=side scatter, FSC=forward scatter, FL1=fluorescence, R1=region of interest 1 (i.e. *S. aureus*), M1=marker indicating area of fluorescence above background level.

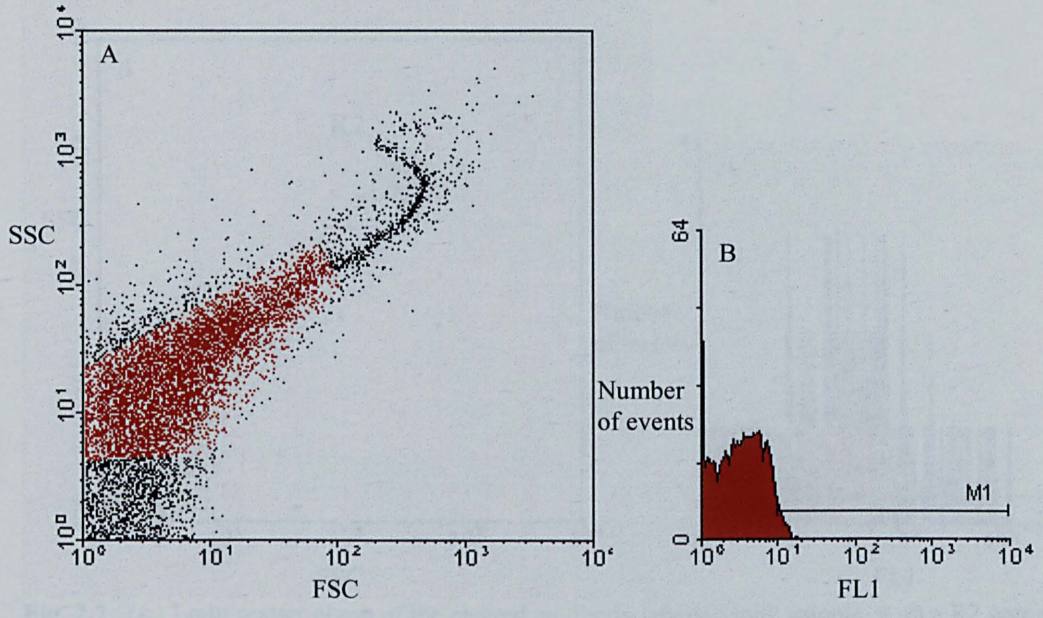


Fig. 2.5. (A) Light scatter graph of cleared (non-labelled) milk sample, with the R1 gate applied and (B) fluorescence histogram of R1.
SSC=side scatter, FSC=forward scatter, FL1=fluorescence, M1=marker indicating area of fluorescence above background level.

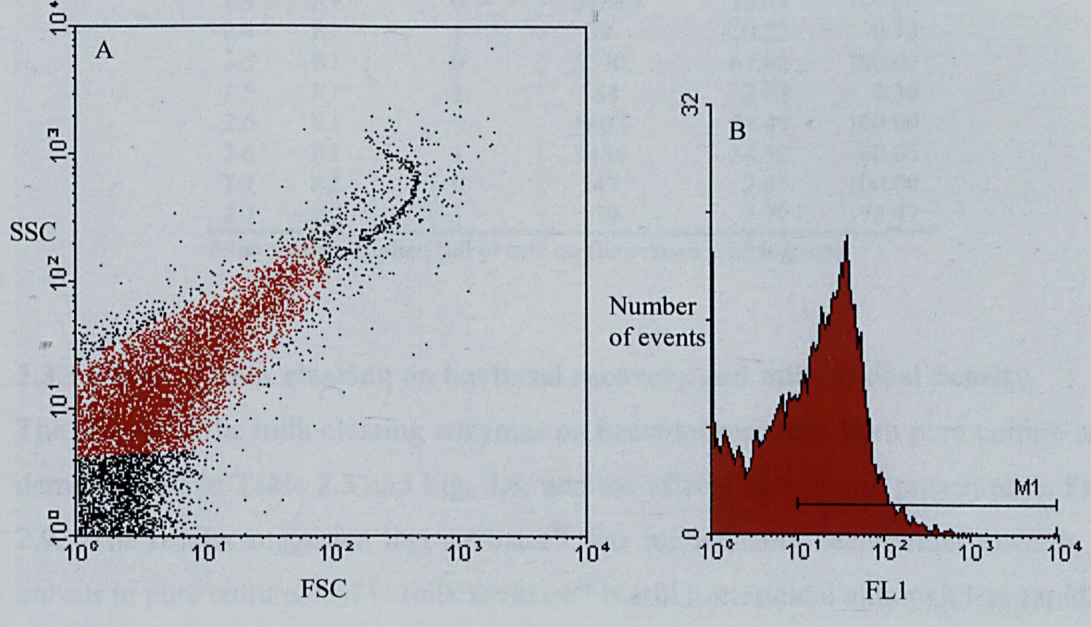


Fig. 2.6. (A) Light scatter graph of the cleared, antibody labelled milk sample with the R1 gate applied and (B) fluorescence histogram of R1.
SSC=side scatter, FSC=forward scatter, FL1=fluorescence, M1=marker indicating area of fluorescence above background level.

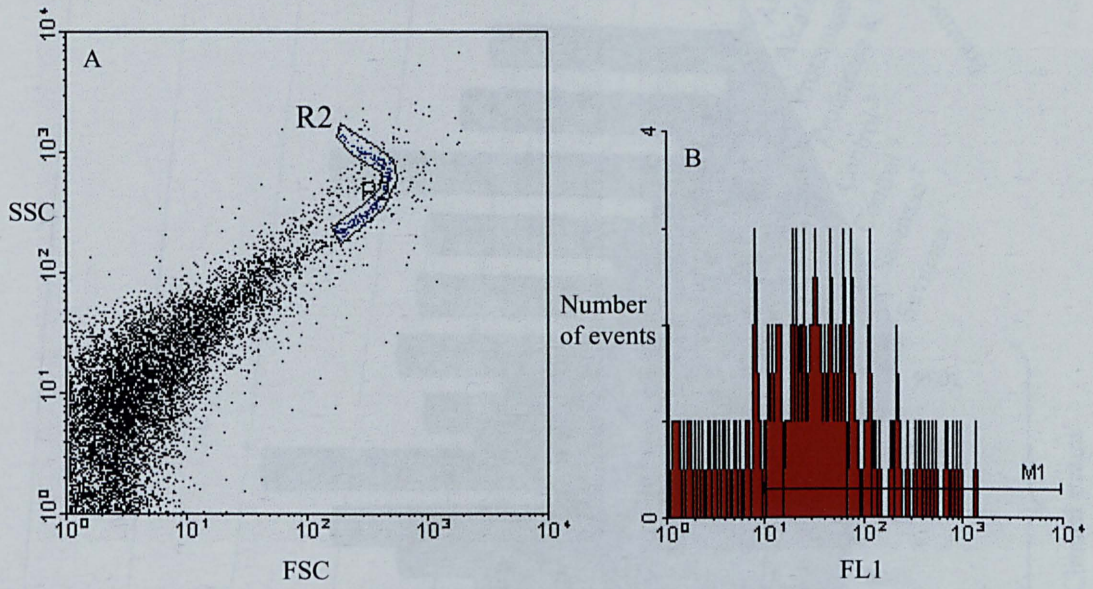


Fig. 2.7. (A) Light scatter graph of the cleared, antibody labelled milk sample, with a R2 gate applied and (B) fluorescence histogram of R2.
SSC=side scatter, FSC=forward scatter, FL1=fluorescence, R2=region of interest 2, M1=marker indicating area of fluorescence above background level.

Table 2.2. Data of events captured for Figs. 2.3-2.7.

Fig.	Gate	Marker (M)	Events	% of total	% gated
2.3	none	0	45022	100.00	100.00
2.3	none	1	241	0.54	0.54
2.4	R1	0	5188	30.64	100.00
2.4	R1	1	39	0.23	0.75
2.5	R1	0	5570	61.02	100.00
2.5	R1	1	184	2.02	3.30
2.6	R1	0	5802	57.49	100.00
2.6	R1	1	3484	34.52	60.05
2.7	R2	0	247	2.45	100.00
2.7	R2	1	179	1.77	72.47

0=no marker applied (all events on fluorescence histogram)

2.3.3 Effect of milk clearing on bacterial recovery and milk optical density

The effects of the milk clearing enzymes on bacterial recovery from pure culture are demonstrated in Table 2.3 and Fig. 2.8, and the effects in milk are presented in Fig. 2.9. The results suggested that savinase® was immediately bactericidal towards *S. aureus* in pure culture, and in milk savinase® is still bactericidal although less rapid.

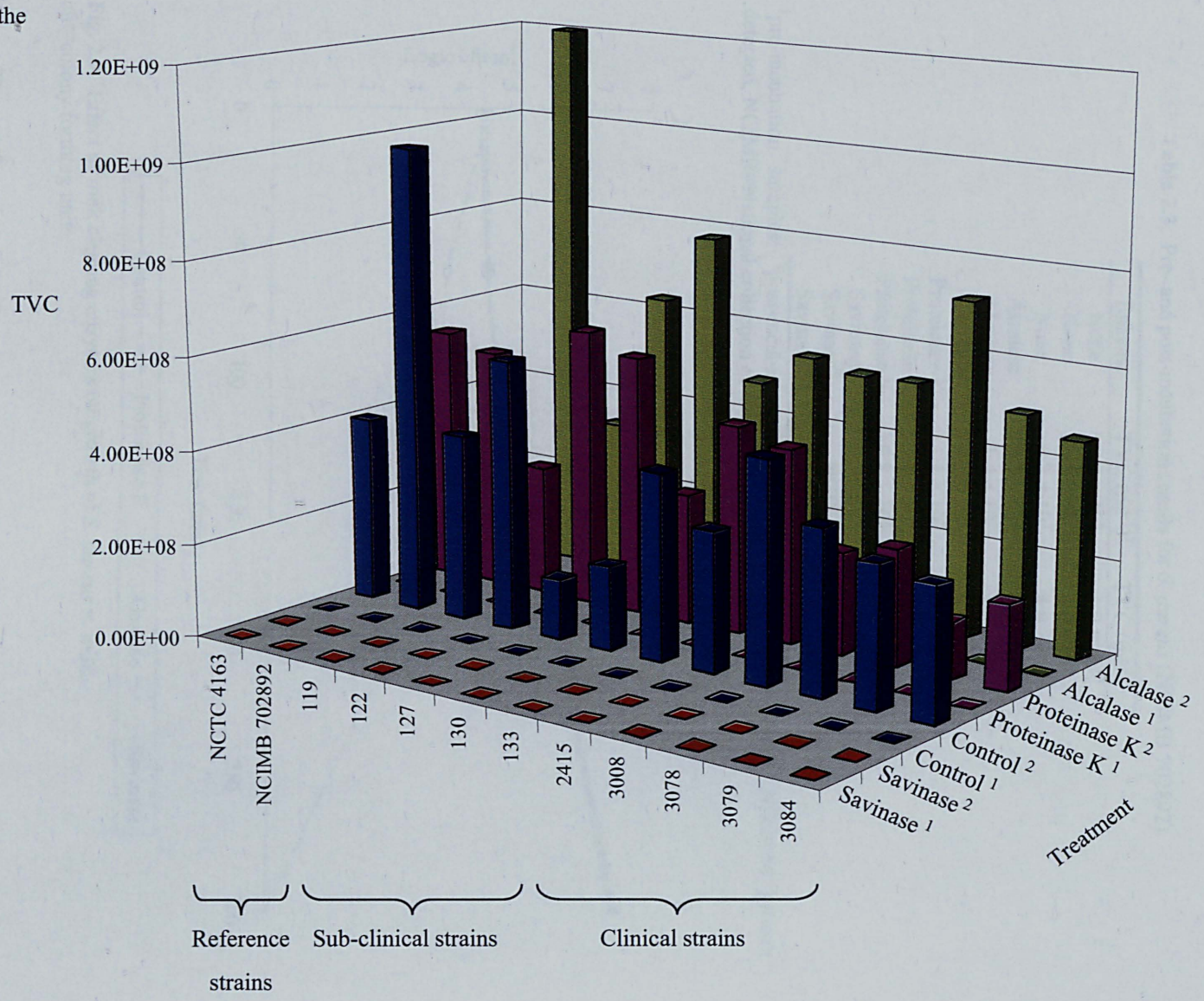


Fig. 2.8. Effect of milk clearing enzymes on the growth of *S. aureus*.

[1] pre-incubation; [2] post-incubation, NCTC=National collection of type cultures, NCIMB=National collection of industrial and marine bacteria.

Table 2.3. Pre- and post-incubation results for *S. aureus* (NCIMB 702892).

Enzyme	TVC (ml^{-1})[1]	TVC (ml^{-1})[2]
None	5.8×10^4	4.0×10^8
None	1.6×10^4	1.0×10^9
None	9.3×10^3	4.0×10^8
Alcalase	1.3×10^5	1.4×10^8
Alcalase	1.4×10^5	1.2×10^9
Alcalase	9.6×10^3	3.3×10^8
Proteinase K	1.1×10^5	5.5×10^8
Proteinase K	1.4×10^5	5.2×10^8
Proteinase K	8.8×10^3	2.8×10^8
Savinase®	NGD	NGD
Savinase®	NGD	NGD
Savinase®	NGD	NGD

[1]pre-incubation samples; [2]post-incubation samples, TVC=total viable count, NGD=no growth detected, NCIMB=National collection of industrial and marine bacteria.

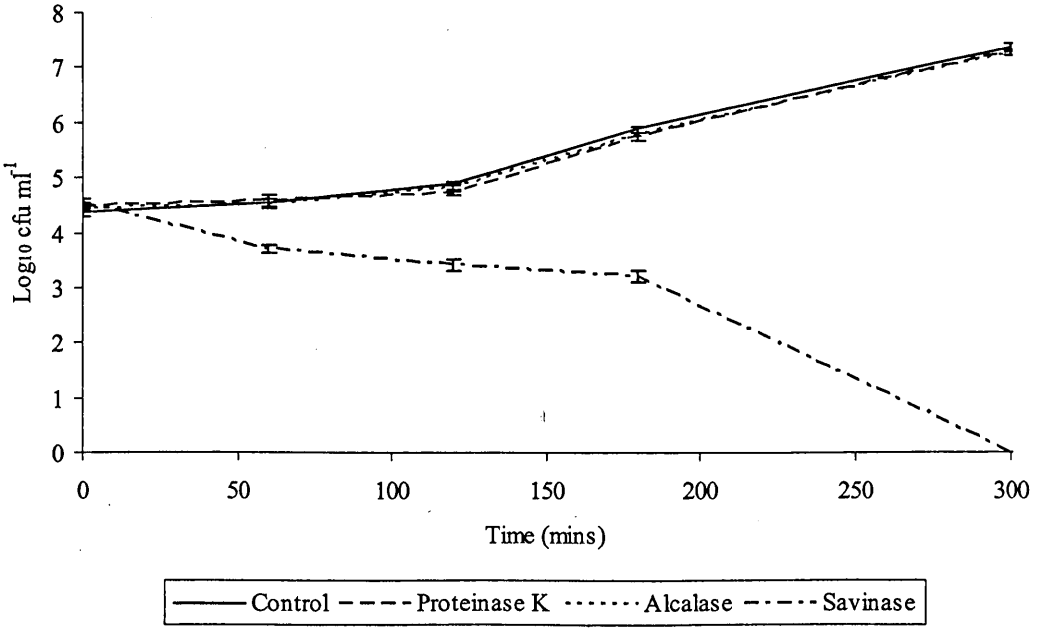


Fig. 2.9. Effect of milk clearing enzymes on growth of *S. aureus* in milk.
cfu=colony forming units.

The effects of each of the enzymes on the OD of milk are presented in Fig. 2.10. Savinase® was more efficient at reducing the OD, but it's effects on bacterial recovery precluded it from use in the final protocol.

The effects of Proteinase K and alcalase® were very similar with respect to bacterial recovery and reducing milk OD, and both are suitable for use in the milk preparation protocol.

2.3.4 Bacterial Staining

Labelled bacteria were detected at the 1µl inclusion level (Fig. 2.11), although the proportion labelled cannot be determined as the phase contrast exposure failed. The 2µl inclusion level (Fig. 2.12) was sufficient to label the majority of bacteria, and demonstrates that whilst the 5 and 10µl levels were able to label the bacteria (Figs. 2.13 and 2.14), this inclusion level was unnecessary.

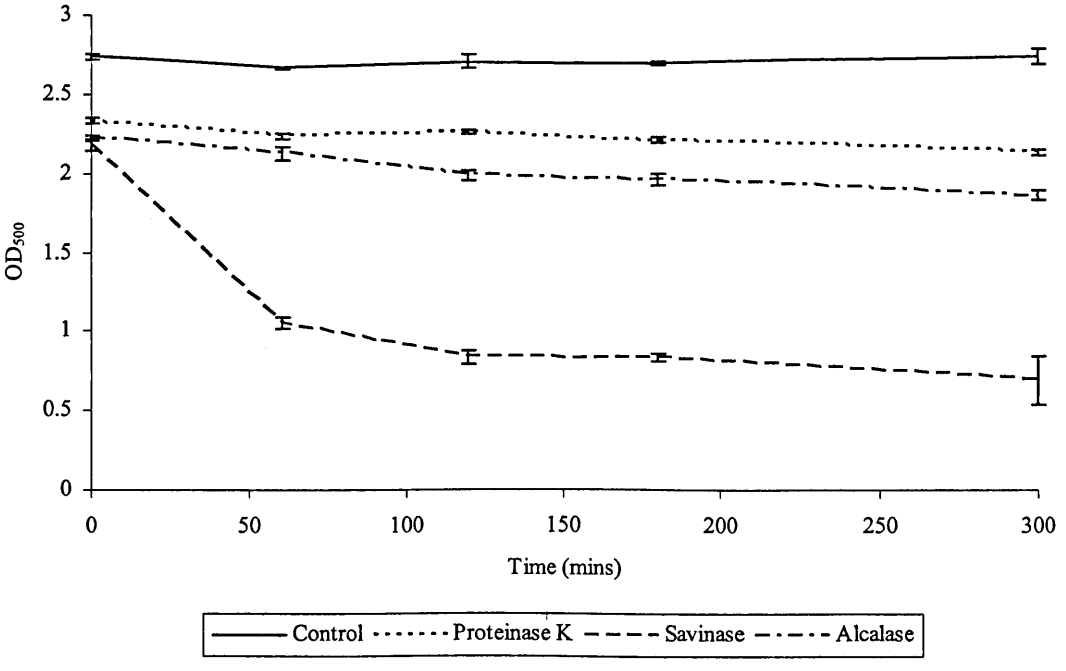


Fig. 2.10. Effect of the milk clearing enzymes on milk optical density.
OD_{500}=optical density at 500nm

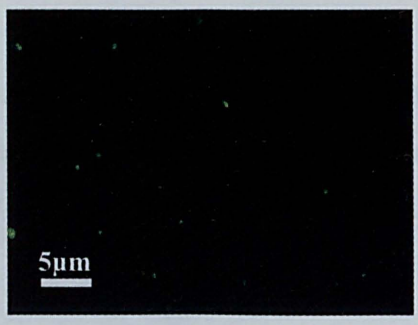


Fig. 2.11. Fluorescent microscopy slide of 1µl antibody labelled suspension.

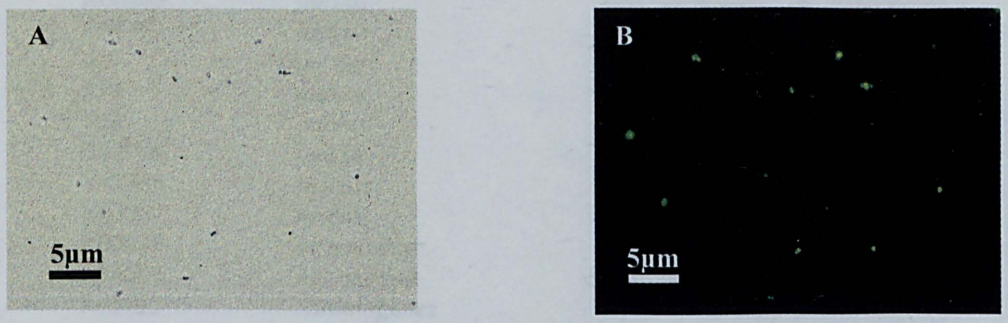


Fig. 2.12. 2µl antibody labelled suspension viewed using (A) phase contrast and (B) fluorescent microscopy.

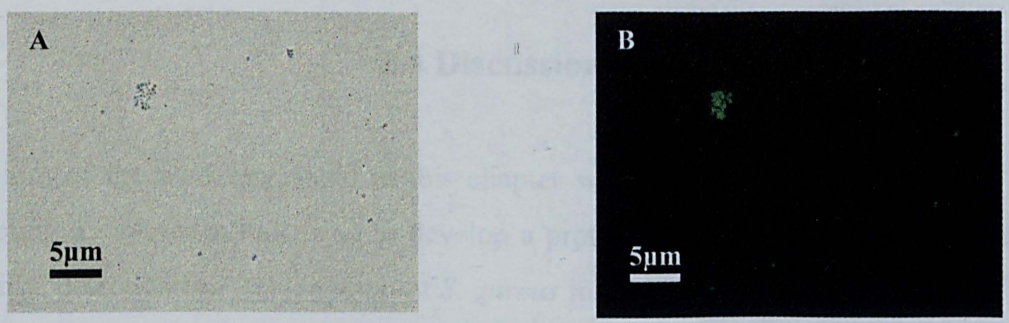


Fig. 2.13. 5µl antibody labelled suspension viewed using (A) phase contrast and (B) fluorescent microscopy.

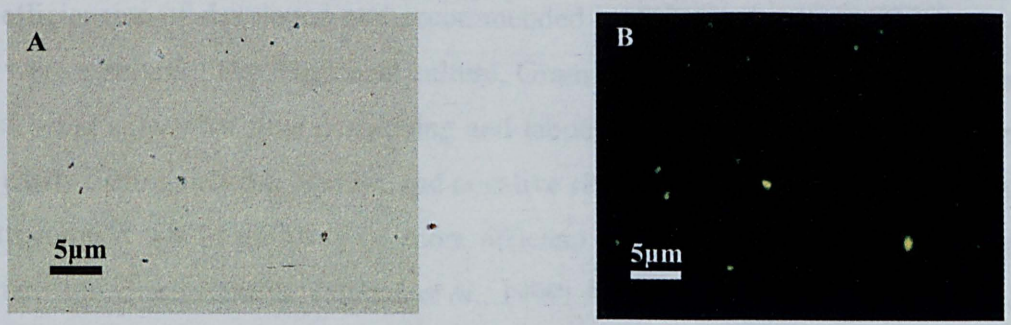


Fig. 2.14. 10µl antibody labelled suspension viewed using (A) phase contrast and (B) fluorescent microscopy.

2.3.5 Antibody specificity

The results of the antibody specificity testing are presented in Table 2.4. The antibody bound to all strains of *S. aureus* tested, however it also bound to coagulase-negative staphylococci (CNS), and coagulase-negative strains of the coagulase variable species *S. hyicus* and *S. intermedius*.

Table 2.4. *S. aureus* antibody specificity testing.

Reference No.	Staphylococcal spp.	Coagulase	Antibody labelled
NCIMB 702892	*aureus*	+	+
R06264	*aureus*	+	+
R06265	*aureus*	+	+
R06557	*aureus*	+	+
119	*aureus*	+	+
130	*aureus*	+	+
133	*aureus*	+	+
B00794	*hyicus*	-	-
Y03245	*hyicus*	-	+
Y03297	*hyicus*	-	+
R00678	*intermedius*	-	+
R04603	*intermedius*	-	-
R05653	*chromogenes*	-	-
ATCC 12228	*epidermidis*	-	+
NCTC 11046	*simulans*	-	-

+=positive reaction, -=negative reaction, spp.=species.

2.4 Discussion

The aim of the work presented in this chapter was to assess different methods of detecting *S. aureus* in milk, and to develop a protocol which allowed the rapid and specific, detection and enumeration of *S. aureus* in milk. This was then to be used as a screening method for milk samples collected as part of a longitudinal study (Chapter 3).

The efficiencies of developed and recommended techniques to detect *S. aureus* in milk were assessed. The traditional culture, Gram stain, catalase and coagulase tests whilst being somewhat time consuming and labour intensive, are simple to perform with easily distinguishable positive and negative reactions. Although in a large scale investigation it would probably be more efficient to use the KOH string test as an alternative to Gram staining, (Hogan *et al.*, 1999; Arthi *et al.*, 2003). Indeed Arthi *et al.* (2003) reported 100 and 98.95% of Gram-positive and -negative bacteria

respectively were correctly identified by the KOH string test. Notwithstanding the length of time taken to obtain a result, these traditional 'gold standard' techniques were able to identify *S. aureus* easily, and sufficiently classified other bacteria in milk.

The API strips are a simple method of identifying unknown organisms, although some previous knowledge of the bacterial family is required to determine which API kit is suitable. The samples tested as part of this project have demonstrated that this method can suffer from a lack of accuracy and reproducibility. Sample 98a was determined to be coagulase-positive by the tube coagulase test (most likely *S. aureus*), but was identified as *S. sciuri*, [a coagulase-negative species] following API classification. Analysis of veterinary as opposed to human, isolates also increased the likelihood of mis-identification, and the API tests offer no timesaving benefit over culture techniques, as colonies were required for testing.

These results demonstrate the limitations of current methods of bacterial identification. The low level of reproducibility encountered with the API strips may be due to analysis of more than one colony per strip. Picking colonies from an agar plate to form a suspension may have resulted in the presence of more than one bacterial spp., leading to varying results, dependent on the spp. selected. The detection of more than one bacterial spp. within a mammary quarter has been demonstrated (Brooks and Barnum, 1984*b*; Nickerson and Boddie, 1994). Indeed, Nickerson and Boddie (1994) were able to demonstrate the presence of coagulase-positive and -negative staphylococcal spp. in the same quarter, and this may go some way to explaining the variable and inconsistent results.

A major advantage of the method developed in this chapter to allow the rapid and specific detection of *S. aureus* in milk is that it analyses one millilitre of milk rather than 10-100µl. This will help the protocol obtain a high level of sensitivity; Buelow *et al.* (1996*a*) reported that the greatest increase in sensitivity for diagnosis of *S. aureus* IMI was achieved by increasing inoculum volume. Also, samples can easily be concentrated to improve sensitivity further, by resuspension in smaller volumes.

The effects of different conditions and reagents were investigated to optimise the bacterial recovery rate. The individual changes had little effect, although the assay maintained a high and robust rate of bacterial recovery, and exceeded that of McClelland and Pinder (1994) who reported a recovery rate of 70-80%.

A protease enzyme was used to allow distinction of the bacteria by FCM. Both proteinase K and savinase® had previously been identified as being suitable (Gunasekera *et al.*, 2000). However, in the UK at least, savinase® was bactericidal towards *S. aureus* in both milk and pure culture (Smith *et al.*, 2003). It is therefore unlikely that savinase® would be suitable for use in an assay that may also be used for viability determination. It has been suggested (F. Valle, *personal communication*) that the variation between the results of Gunasekera and colleagues (*reply to*: Smith *et al.*, 2003) and those determined during the course of this work, may be due to the production of antimicrobial peptide(s) in the UK milk samples by savinase®. It could be that these peptides were then bactericidal toward *S. aureus* and not savinase®. Whilst this may explain the discrepancy between the results, tests in pure culture support the theory that savinase® is bactericidal toward *S. aureus*.

Fluorescent antibody labelling was used to label *S. aureus*, and a final antibody inclusion level of 2µl proved to be sufficient to label the majority of bacteria in a sample. However antibody labelling lacked specificity and, in the present study, could not have been used to diagnose intra-mammary infections (IMI) caused by *S. aureus* or all staphylococcal spp. Further evaluation of alternative labelling methods is required. One suitable alternative, which allows the labelling of specific nucleic acid sequences inside intact cells, is fluorescent *in situ* hybridisation (FISH) (Ruimy *et al.*, 1994; Deere *et al.*, 1998; Hogardt *et al.*, 2000; Veal *et al.*, 2000; Zoetendal *et al.*, 2002; Gunasekera *et al.*, 2003; Oliveira *et al.*, 2003). Cells are fixed and permeabilised to permit entry of an oligonucleotide probe, conditions of probe binding and washing are then selected so that the probe will bind only to its target rRNA sequence. Labelled cells can then be visualised by fluorescent microscopy or FCM (Veal *et al.*, 2000). FISH offers the advantage of not destroying the target cells and as fluorescence is conferred by an oligonucleotide probe, this correlates to cellular rRNA content which in turn relates to viability (Ruimy *et al.*, 1994; Veal *et al.*, 2000), removing the need for analysis of an additional sample. FISH methods

have been developed to detect *S. aureus* from clinical samples obtained from cystic fibrosis patients (Hogardt *et al.*, 2000) and *Listeria monocytogenes* and *Pseudomonas* spp. in milk (Gunasekera *et al.*, 2003; Oliveira *et al.*, 2003).

Ultimately however, PCR-based techniques (Martinez *et al.*, 2001; Meiri-Bendek *et al.*, 2002; Phuektes *et al.*, 2001*a*, 2003; Riffon *et al.*, 2001) of diagnosing IMI may be used more for individual samples, with FCM reserved for bulk milk analyses. This is because PCR-based techniques already have a much lower sensitivity than FCM, and require much less investment.

2.5 Conclusions and further work

The method developed as part of this project involved incubating a milk sample with 10% (v/v) alcalase® (Novozymes) at 37°C for 45min, followed by centrifugation at 10,000×*g* for 10min and then removal of the supernatant, fats and digested proteins. The pellet was then resuspended in 0.5% (w/v) BSA ready for labelling.

The method is suitable for detecting micro-organisms in milk, and it is likely that the protocol could be optimised for the majority of bacterial species found in milk. This would offer advantages of speed of detection, and diagnosis over the currently employed cultural techniques.

However the technique still requires calibration to ensure robustness and reliability. The lower detection limit of the assay also needs to be determined. The assay could be used for analysis of individual milk samples, as a bacterial concentration of 10^4 *S. aureus* ml^{-1} was detectable with no enrichment or sample concentration, and further enhancement would be likely to lower this limit.

However, the main drawback to the method at present is the lack of a suitably specific bacterial labelling protocol, and this area requires further attention. The sensitivity of the MAb used was excellent as all *S. aureus* isolates tested were

labelled, but the specificity was poor as other staphylococci were labelled in addition to *S. aureus*.

Chapter 3: Investigation of *Staphylcoccus aureus* on an organic dairy farm

3.1 Introduction

Mastitis is the most frequently occurring health problem in organic dairy herds (Weller and Bowling, 2000), and the most frequently isolated pathogens are *S. aureus* and *Str. uberis* (Vaarst and Enevoldsen, 1997; Busato *et al.*, 2000; Weller and Bowling, 2000; Barlow, 2001 Turner, 2001). Control of contagious mastitis is made more difficult on organic compared to conventional dairies without the routine use of dry cow therapy (DCT) at the end of lactation (Hovi and Roderick, 1998).

Organic differs from conventional farming in methods of land and animal management. Soil richness and fertility is maintained by restoring organic matter and avoiding synthetic fertilisers, pesticides and herbicides. Homeopathy and herbal remedies are used routinely in the management of livestock instead of antibiotics and other drugs, although in cases of acute illness conventional drug treatments are used where the animal might otherwise suffer (http://www.soilassociation.org).

The traditional view of *S. aureus* mastitis is that lactating mammary glands form the reservoir of infection (Davidson, 1961; Newbould, 1968), although environmental *S. aureus* isolates have been detected on dairy farms in the USA (Fox *et al.*, 1991; Matos *et al.*, 1991; Roberson *et al.*, 1994*b*). Similar research from the UK and particularly on organic dairy farms worldwide is unknown. Thus detection of potential sources of infection is important in developing effective control mechanisms.

The milk and environment of an organic dairy farm was extensively sampled to test the hypothesis that the environment may form an important reservoir of *S. aureus* infection for an organic dairy herd in the UK.

The aim of the work presented in this chapter was twofold; first, to isolate coagulase-positive staphylococci (CPS) (particularly *S. aureus*) from the milk and environment of an organic dairy cattle farm for strain typing and analysis (Chapter 4); second, to

carry out a longitudinal observational study of infections in cows and heifers, and the environment.

3.2 Materials and methods

3.2.1 Herd selection

In November 2001 an organic farmer in Somerset was contacted and asked whether he knew of any herds with *S. aureus* infections. The following month the same farmer experienced a *S. aureus* mastitis problem, welcomed an investigation, and it was felt that there would be a high level of interest and compliance in the study.

A herd of 100 (increasing to 150) Channel Island breed dairy cattle completed its conversion to full organic status in the summer of 1999. The herd was milked twice a day through a 5/10 herringbone parlour, with an annual milk yield of 5-6,000kg/head. Homeopathy and non-antibiotic therapy were the treatments for mastitis. The herd was cubicle housed over winter and fed a silage-based ration, supplemented by the use of in-parlour feeders. Over summer the herd was at pasture.

Individual cow somatic cell counts (ICSCC) were monitored monthly by the farm. Cases of clinical mastitis and high cell counts were treated with homeopathic remedies e.g. *Calcarea fluorica* and *Hepar sulphuris* (Vermeulen, 1996)

3.2.2 Sampling period and animal selection

Samples were collected from December 2001 to September 2002. At the start, 15 cows were selected based on their ICSCC results. The cows had a mean SCC of 530,000 cells ml^{-1} (range 120–1,150,000). Cows were also recruited into the study when their ICSCC (determined by the farms monitoring programme) rose above 400,000 cells ml^{-1} for two consecutive months.

A group of Devon-cross in-calf heifers were due to join the milking herd as they calved and began their first lactation. Body sites of these animals were sampled before they joined the herd, and quarter milk samples were collected each month

once they came into lactation. This sampling pattern continued to the end of the study, although heifer body sites were not sampled after three months of lactation, to prevent stress.

3.2.3 Sampling procedures

Sterilised latex examination gloves were worn for the collection of all samples.

Collection of milk samples: On the first visit, milk samples were collected before the cows were milked, udders were cleaned as usual by the milking staff, the teats were then scrubbed with 70% (v/v) ethanol and allowed to dry. The first few streams of milk were discarded, and individual quarter samples were collected into pre-labelled universal tubes (Greiner Bio-One Ltd., Stonehouse, UK).

At all subsequent visits, post-milking milk samples were taken. Teats were initially dipped in an iodine based pre-milking teat dip (Total Control Dip, EmpraSan (Chemicals) Ltd, Birkenhead, UK), and a contact time of at least 30s was allowed before teats were dried with individual paper towels. Teats were then scrubbed with a cotton wool ball moistened with 70% (v/v) ethanol, and allowed to dry before the sample was collected. Again the first few streams of milk were discarded and samples were collected into pre-labelled universal tubes (Greiner Bio-One Ltd.). The cows and heifers were restricted to the left-hand side of the parlour, and quarters were sampled in the order left fore (LF), left hind (LH), right fore (RF) and right hind (RH). Following sample collection, teats were dipped in the farm's own iodine-based post-milking teat disinfectant before the cows and heifers were released.

Samples for commercial somatic cell counting were taken pre-milking after the usual udder preparation by the milking personnel. Samples were collected approximately filling ½-¾ of the container provided (National Milk Records plc (NMR) Chippenham, Wiltshire, UK) which contained a few drops of the preservative 2-bromo-2-nitropropane-1,3-diol (Bronopol). A record was made of the container number used for each quarter of each heifer and samples were sent to the NMR laboratory for analysis using the freepost packaging provided. Results were returned within two days of sample collection.

Body Site Samples: Body site samples were taken from in-calf heifers before they entered the milking herd, and up to 12 weeks of lactation. All samples were taken using transport swabs (SW 350 or SW 351; Appleton Woods Ltd., Selly Oak, UK) and stored in amies transport medium with (SW 351) or without charcoal (SW 350).

Teat skin samples: A sampling method which included both teat side and orifice on the same swab was employed. The swab was initially moistened in 0.5ml of sterile tryptone soya broth (TSB) (Oxoid, Basingstoke, UK) to facilitate collection and maintenance of viable bacteria from a dry surface, then wiped as illustrated (Fig. 3.1), across the teat and orifice before it was stored in transport medium. Dirt was removed from heavily soiled teats before the sample was collected with a dry paper towel.

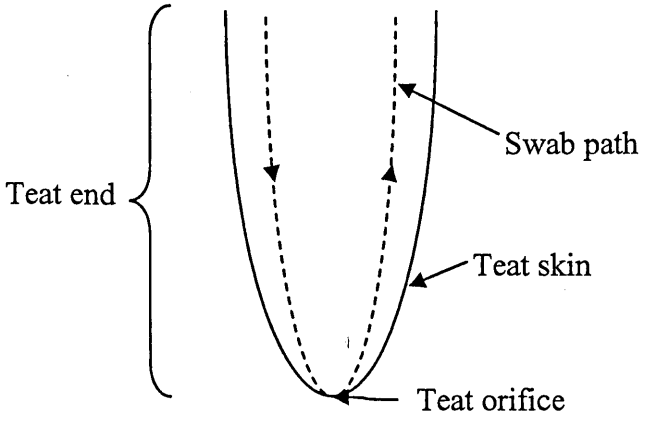


Fig 3.1. Teat skin and orifice swabbing technique.

Vaginal and muzzle samples: There was no need to moisten the swabs. To take the vaginal sample, the heifer was restrained in the crush and the lips of the vulva were parted, the swab inserted and rubbed on the mucosal surfaces. Heavily soiled vulvas were cleaned prior to sample collection using a ball of cotton wool soaked in 70% (v/v) ethanol. To take the muzzle sample, the swab was inserted into the muzzle and inner nares and rubbed against the mucosal surfaces.

Bedding and foodstuff samples: Bedding was collected from shallow, deep, dry and damp areas to ensure that the samples were representative of the environment. Foodstuffs were collected in the same manner from both the storage e.g. silage clamp face, and feeding areas.

All bedding and foodstuff samples were collected into large (300mm×539mm) freezer bags (Safeway Stores plc, Hayes, UK), and stored frozen at –20°C for up to three weeks, until there was time to process them.

Housing and equipment samples: Samples of housing, animal handling, and feeding equipment surfaces were taken using transport swabs as described for teat skin samples. Sample sites included cubicle partitions, the crush, feed faces, ring feeders and feeding troughs.

Parlour floor: The area of the floor along the full length of the parlour (both sides) beneath the cow's and heifer's udders was sampled with an unmoistened transport swab (Appleton Woods Ltd.).

Parlour gates: The gates on either side of the milking parlour were sampled using a moistened transport swab (Appleton Woods Ltd.).

Milking machine clusters: All milking machine clusters (five 'normal' clusters, one used for high cell count cows and one with smaller liners used for Jersey heifers) were sampled using an unmoistened transport swab at the end of milking.

Air samples: Air samples were obtained by exposing a modified Baird Parker (MBP) (Oxoid) agar plate for 1h. Plates were sealed using Nescofilm (Nippon Shoji Kaisha Ltd, Osaka, Japan) for transport. Sample sites included the parlour at work and rest, dry cow, lactating cow and suckler cow housing and the calving box.

Water samples: Water samples were collected into sterile universal containers (Greiner Bio-One Ltd.) by opening them inverted, 5-10ml of water was scooped into them and they were sealed immediately. Sample sites included all drinking water sources, parlour wash water, udder wash water and the high cell count cluster wash water.

Milking personnel: All milking personnel (and myself) were sampled during the milking process. Transport swabs (Appleton Woods Ltd.) were used to sample the

hands and nares. Swabs were moistened in 0.5ml sterile TSB (Oxoid) for hand samples, and left dry for sampling the nares.

Non-bovine animals: All dogs (and cats when possible) had the mucosal surfaces of their mouths sampled with a transport swab (Appleton Woods Ltd.).

Once the sampling was completed, all samples were stored on ice for return to the laboratory.

3.2.4 Bacteriological Procedures

Somatic Cell Counts (SCC): Samples were submitted to a commercial company (NMR) for automatic somatic cell counting using a Fossomatic 360.

Milk sample bacteriology: Samples were analysed according to the recommendations of the National Mastitis Council (NMC; Hogan *et al.*, 1999), bacteriological identification was carried out as follows:

Milk samples were thawed at room temperature. One-hundred microlitres was spread across a sheep's blood agar (SBA) plate containing 5% (v/v) sterile sheep's blood. Plates were incubated inverted at 37°C and checked at 24 and 48h.

If three or more distinct colony types were isolated, the sample was classed as contaminated and discarded. At 24h (and 48h if new colonies had grown) individual colony types were sub-cultured onto one quadrant of a 5% (v/v) SBA plate (Oxoid) and incubated at 37°C overnight to provide sufficient culture for biochemical tests. The number of colonies of distinct types was estimated to give information on the number of colony forming units (cfu) ml^{-1} in the original sample.

Colonies were identified based on their morphology on 5% (v/v) SBA, and reaction to KOH string, catalase and tube coagulase tests (BD, Oxford, UK) (Chapter 2; Fig. 2.2), and the presence or absence of growth on MBP agar (Oxoid).

Cultures which gave a positive KOH string test were classed as Gram-negative, KOH-negative cultures were further identified using the catalase test. Catalase-

negative cultures were classed as streptococci. KOH-negative, catalase-positive cultures included corynebacteria and staphylococci, differentiation was based on colony morphology. Corynebacteria were stored and the staphylococci were tested for coagulase production using the tube coagulase test (TCT; BD) and checked after 1, 4, and 24h. All TCT-negative cultures were classed as coagulase-negative staphylococci (CNS) and stored, all positive cultures were streaked across MBP agar (Oxoid) to differentiate *S. aureus* from other coagulase-positive staphylococci i.e. *S. hyicus* and *S. intermedius*.

Milking machine cluster samples: These were streaked across one half of a 5% (v/v) SBA (Oxoid) plate, incubated inverted at 37°C, and processed as described for milk samples.

Body site, housing, equipment, milking personnel, non-bovine animals, parlour floor and gates, air and water samples: Swabs were streaked across one quadrant of a MBP agar (Oxoid) plate and 100µl of each water sample were spread across the surface of an MBP plate. All MBP plates, including air samples, were incubated at 37°C for 24-48h.

Suspect *S. aureus* colonies were streaked across one quadrant of a 5% (v/v) SBA plate (Oxoid) for biochemical tests. Colonies were identified using the procedures described for milk samples.

Bedding and foodstuff samples: Bacteriological analyses of feed and bedding were carried out using the methods of Hutton *et al.* (1990) and Roberson *et al.* (1994*b*) with modifications, as described below.

Samples were defrosted at room temperature and chopped into approximately 50mm lengths using a pair of sterilised scissors. Ten grams were added to 100ml sterile TSB (Oxoid) in a 250ml conical flask and placed in a rotary shaker (Gallenkamp Orbital Incubator, SANYO Gallenkamp Plc., Loughborough, UK). Samples were shaken at 120rpm for 30min at room temperature. Serial 10-fold dilutions were made of the resulting broth, to a final dilution of 1:1000. One hundred microlitres (0.1ml) of the 1:10, 1:100 and 1:1000 dilutions were spread across the surface of a

MBP agar (Oxoid) plate and incubated inverted at 37°C for 24-48h. Colony identification was carried out as described for body site samples.

Following analysis, samples were stored at –20°C and retained cultures were stored in 30% glycerol TSB (Oxoid) at –80°C.

3.2.5 Data storage and analysis

After the completion of each month's sample analyses, results were entered into a Microsoft Excel (Excel 97: Microsoft Ltd., Reading, UK) spreadsheet. Data analysis was carried out in Excel (Excel 97 and 2000: Microsoft Ltd.), and SPSS (SPSS 10.0 Client/Server, Release 10.0.5.336: SPSS Inc., Chicago ILL). Statistical analysis of the data was performed using Chi-Square tables (Epi Info version 6.04d: Centers for Disease Control and Prevention (CDC), USA).

Seasonal effects were investigated by classifying visits 1-3 (Dec–Feb) as winter, 4-6 (Mar–May) as spring and 7-10 (Jun-Sept) as summer.

Kaplan-Meier survival curves were constructed after calculating the duration (in days) of all quarter infections, and determining whether the infection had been cleared or the data censored (see section 3.2.6). Data were stored in MS Excel (Microsoft Ltd.), analysed and presented in SPSS (SPSS). Curves were presented with survival function on the Y-axis, a value of one was equal to 100% infection survival, and 0.1 indicated that 10% of infections remained. A survival function of 0.5 (50%) indicated the median time to recovery from infection.

3.2.6 Case definitions

Infection: The detection of an organism in the milk sample collected.

Persistent infection: The same species isolated from the same quarter at successive visits.

New infection: If the species detected had not been detected in the same quarter at the previous visit.

Recovered/Cleared: If an organism that had been detected at one visit was not isolated from the same quarter at the following visit.

Censored: Data collection ended before the infection cleared e.g. due to culling, drying-off or no further samples were taken.

Clinical mastitis: The presence of visibly detectable physical changes in milk or the udder e.g. clots in milk or hot/swollen udder.

Subclinical mastitis: No physical evidence of infection.

3.3 Results

3.3.1 Bacteria isolated from milk

The average sampling interval was 29 days (range 28–36) and 2101 samples were collected for analysis. This ranged from 97–347 per visit with an average of 210. There were 959 (45.6%) quarter milk samples for bacteriology and 420 (20.0%) milk samples were submitted for commercial somatic cell counting (Table 3.1). The remaining 722 (34.4%) samples came from the environment and body sites (Appendix 3). In total 1681 samples were collected for bacteriological analysis (Table 3.2).

The 959 quarter milk samples for bacteriology consisted of 499 samples from cows, 422 samples taken from the bought-in heifers, 34 from cattle at calving and four clinical mastitis cases collected by the farm staff (Table 3.1). Eleven milk samples were contaminated, all were from cows, and ten (90.9%) occurred in the first two visits (Table 3.1).

Twenty-six cows were sampled, with a range of 1–10 samples collected per quarter. The mean lactation number was 4 (range 1–8). The 21 heifers were sampled an average [median] of 5 times (range 2 – 6).

There were 488 non-contaminated cow quarter milk samples, 29.1% (142) contained coagulase-positive staphylococci (CPS). No growth was detected in 36.9% (180), and coagulase-negative staphylococci (CNS), streptococci, corynebacteria and Gram-negative bacteria were detected in 25.0, 8.2, 5.9 and 4.7% (122, 40, 29 and 23) of samples respectively.

Of the 422 heifer quarter milk samples, none were contaminated and no CPS growth was detected. No growth was detected in 58.5% (247) and CNS, Gram-negative bacteria, streptococci, and corynebacteria were detected in 34.6, 5.7, 5.5 and 0.2% (146, 24, 23, and 1) of samples respectively.

Table 3.1. Number of quarter milk samples and number with each organism detected by visit.

	Visit number										
Milk sample/organism	1	2	3	4	5	6	7	8	9	10	Total (%)
Bacteriology: Cow	**60**	**32**	**12**	**28**	**51**	**51**	**79**	**63**	**67**	**56**	**499**
CPS	20	15	4	6	11	14	18	17	20	17	142 (29.1)[1]
CNS	8	5	5	5	12	18	24	13	12	20	122 (25.0)[1]
Streptococci	5	7	0	2	9	5	3	4	4	1	40 (8.2)[1]
Corynebacteria	0	1	1	0	0	0	5	7	8	7	29 (5.9)[1]
Gram-negative	5	3	0	3	2	0	7	2	1	0	23 (4.7)[1]
NGD	28	7	3	15	22	15	29	20	25	16	180 (36.9)[1]
Contaminated	4	6	0	0	1	0	0	0	0	0	11 (2.2)
Calving	**0**	**0**	**30**	**4**	**0**	**0**	**0**	**0**	**0**	**0**	**34**
CNS			17	1							18 (52.9)
Streptococci			6	0							6 (17.6)
Gram-negative			1	1							2 (5.9)
NGD			7	3							10 (29.4)
Clinical	**0**	**0**	**4**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**4**
Streptococci			1								1 (25.0)
NGD			3								3 (75.0)
Bacteriology: Heifer	**0**	**0**	**0**	**0**	**24**	**72**	**80**	**80**	**83**	**83**	**422**
CPS					0	0	0	0	0	0	0 (0.0)
CNS					5	27	29	21	28	36	146 (34.6)
Streptococci					1	2	1	7	6	6	23 (5.5)
Corynebacteria					0	0	0	1	0	0	1 (0.2)
Gram-negative					1	3	6	4	3	7	24 (5.7)
NGD					18	43	46	49	49	42	247 (58.5)
Contaminated					0	0	0	0	0	0	0 (0.0)
SCC	**0**	**0**	**0**	**0**	**23[2]**	**72[2]**	**80[2]**	**79[2]**	**83[2]**	**83[2]**	**420**

[1]percentages calculated on corrected figure of 488 milk samples (total minus contaminated).
[2]samples collected from heifers.
CPS=coagulase-positive staphylococci, CNS=coagulase-negative staphylococci, NGD=no growth detected, SCC=somatic cell count.

The 34 calving and four clinical milk samples submitted by the farm staff yielded 18 (47.4%) CNS, seven (18.4%) streptococci, two (5.3%) Gram-negative bacteria and no growth in 13 (34.2%) of the samples.

3.3.2 Bacterial distribution by quarter

Cows: The distribution of bacteria detected in each quarter (LF, LH, RF and RH) is presented in Table 3.3. Bacterial isolation rates varied for the individual quarters, and the hind quarters had a significantly higher level of overall infection than the fore quarters (χ^2=8.98, $p<0.05$).

Table 3.2. Number (%) of bacteria isolated from each site.

		Number of samples positive for:				
Sample	No. collected	CPS	CNS	Strep.	Coryne.	Gram-neg.
Milk for bact.	**959**	142 (14.8)	286 (29.8)	60 (6.3)	30 (3.1)	49 (5.1)
Clusters	**50**	5 (10.0)	31 (62.0)	12 (24.0)	0 (0.0)	21 (42.0)
Water	**46**	0 (0.0)	14 (30.4)	3 (6.5)	0 (0.0)	21 (45.7)
Environment	**152**	1 (0.7)	0 (0.0)	3 (2.0)	0 (0.0)	0 (0.0)
Non-bovine an.	**20**	2 (10.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Farm personnel	**46**	4 (8.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Heifer body sites	**318**	0 (0.0)	0 (0.0)	2 (0.6)	0 (0.0)	0 (0.0)
Air	**25**	0 (0.0)	16 (64.0)	0 (0.0)	0 (0.0)	1 (4.0)
Feed	**35**	0 (0.0)	0 (0.0)	3 (8.6)	0 (0.0)	0 (0.0)
Bedding	**30**	0 (0.0)	0 (0.0)	2 (6.7)	0 (0.0)	0 (0.0)
TOTAL	**1681**	154 (9.2)	347 (20.6)	85 (5.1)	30 (1.8)	92 (5.5)

CPS=coagulase-positive staphylococci, CNS=coagulase-negative staphylococci, Strep.=streptococci, Coryne.=corynebacteria, Gram-neg.=Gram-negative bacteria, Milk for bact.=milk samples collected for bacteriology, Non-bovine an.=non-bovine animals.

CPS were the most frequently isolated bacteria from the cows and this ranged from 9.5% to 43.8% for individual quarters. Significantly fewer (χ^2=28.45, $p<0.001$) were detected in the LF quarter compared to the other quarters. Consequently significantly more (χ^2=14.53, $p<0.01$) CPS were detected in the hind quarters compared to the fore quarters. CNS were detected in 15.1% of RF to 32.5% of LF quarters. Forty streptococci were detected in the cows milk samples, with an isolation rate of 8.0%, ranging from 1.6% (RF) to 12.7% (LF). Gram-negative bacteria were isolated least often at a rate of 4.6%, ranging from 1.6% (RF) to 6.4% (LF and RR). Corynebacteria were isolated at a rate of 5.8%, this ranged from 1.7% (LH) to 10.3% (LF) for individual quarters. The largest group of samples however, were bacteriologically negative (36.3%). Rates for individual quarters ranged from 22.3% (LH) to 50.8% (RF), with these quarters differing significantly (χ^2=20.31, $p<0.01$).

Table 3.3. Number (%) of cow quarters with each group of bacteria or bacteriologically negative.

Quarter	CPS	CNS	Strep.	Cor.	G-neg.	NGD
Left fore	12 (9.5)^{a}	41 (32.5)^{a}	16 (12.7)^{a}	13 (10.3)^{a}	8 (6.4)	44 (34.9)^{a}
Left hind	53 (43.8)^{b}	34 (28.1)^{a}	10 (8.3)^{a}	2 (1.7)^{b}	5 (4.1)	27 (22.3)^{b}
Right fore	40 (31.8)^{b,c}	19 (15.1)^{b}	2 (1.6)^{b}	4 (3.2)^{b,c}	2 (1.6)	64 (50.8)^{c}
Right hind	37 (29.4)^{c}	27 (21.4)^{a,b}	12 (9.5)^{a}	10 (7.9)^{a,c}	8 (6.4)	46 (36.5)^{a}
Total	142 (28.5)	121 (24.2)	40 (8.0)	29 (5.8)	23 (4.6)	181 (36.3)

^{a,b,c}Figures in the same column with different superscripts differ sugnificantly ($p<0.05$).
CPS=coagulase-positive staphylococci, CNS=coagulase-negative staphylococci, Strep.=streptococci, Cor.=corynebacteria, G-neg.=Gram-negative bacteria, NGD=no growth detected.

Heifers: The distribution of bacteria detected in heifer quarters (LF, LH, RF and RH) is displayed in Table 3.4. There were no statistically significant differences in the rates of isolation of bacteria for individual, or fore and rear quarters.

Table 3.4. Number (%) of heifer quarters with each group of bacteria or bacteriologically negative.

Quarter	CPS	CNS	Strep.	Cor.	G-neg.	NGD
Left front	0 (0.0)	29 (27.9)	8 (7.7)	1 (1.0)	4 (3.9)	65 (62.5)
Left hind	0 (0.0)	40 (37.7)	6 (5.7)	0 (0.0)	7 (6.6)	59 (55.7)
right front	0 (0.0)	41 (38.7)	4 (3.8)	0 (0.0)	8 (7.6)	60 (56.6)
Right hind	0 (0.0)	36 (34.0)	5 (4.7)	0 (0.0)	5 (4.7)	63 (59.4)
Total	0 (0.0)	146 (34.6)	23 (5.5)	1 (0.2)	24 (5.7)	247 (58.5)

CPS=coagulase-positive staphylococci, CNS=coagulase-negative staphylococci, Strep.=streptococci, Cor.=corynebacteria, G-neg.=Gram-negative bacteria, NGD=no growth detected.

CNS were the most frequently isolated bacteria from heifers, the rate ranged from 27.9% to 38.7% for individual quarters. The rate of streptococcal detection ranged from 3.8% (RF) to 7.7% (LF). Streptococcal isolation increased after the first three months of study (2.3-7.7%), and significantly more (χ^2=4.90, $p<0.05$) were detected between visits eight and ten than five and seven. The isolation rate of Gram-negative bacteria ranged from 3.8% to 7.5%, although the majority of heifer samples were bacteriologically negative, and rates for individual quarters ranged from 55.7% (LH) to 62.5% (LF).

Comparison of cow and heifer bacterial isolation rates demonstrated that significantly fewer (χ^2=11.4, $p<0.01$) cow quarters were infected with CNS, and that heifer quarters were more likely to be culture negative (χ^2=44.65, $p<0.01$).

3.3.3 New infection rates

One hundred and ninety six new infections were detected, their distribution by stage of lactation and season is presented in Tables 3.5 and 3.6. Rates of new infections did not vary throughout lactation, although there was a non-significant tendency for a reduction in new infections as lactation progressed. A significantly greater percentage of new infections were detected in the winter and spring compared to the summer (χ^2=14.30, $p<0.001$).

There was no statistically significant difference in the rate of new CPS or CNS infections throughout lactation (Table 3.7), although significantly more new CPS

infections were detected in the winter than the spring or summer (χ^2=10.58, p<0.05; Table 3.8). Conversely significantly fewer CNS infections were detected in winter compared to spring and summer (χ^2=11.66, p<0.001; Table 3.8).

Table 3.5. Number (%) of new infections at the given number of days in milk.

DIM	No. samples	New infections
<100	185	81 (43.8)
100-200	186	71 (38.2)
200+	128	44 (34.4)

DIM = days in milk.

Table 3.6. Number (%) of new infections in different seasons.

Season	No. samples	New infections
Winter	104	58 (55.8)[a]
Spring	130	55 (42.3)[a]
Summer	265	83 (31.3)[b]

Values in the same column with different superscripts differ significantly (p<0.05).

Table 3.7. Number (%) of new CPS and CNS infections at the given DIM.

DIM	New inf.	CPS	CNS
<100	81	22 (27.2)	35 (43.2)
100-200	71	16 (22.5)	33 (46.5)
200+	44	11 (25.0)	14 (31.8)

DIM=days in milk, New inf.=new infections, CPS=coagulase-positive staphylococci, CNS=coagulase-negative staphylococci.

Table 3.8. Number (%) of new CPS and CNS infections in different seasons.

Season	New inf.	CPS	CNS
Winter	58	24 (41.4)[a]	13 (22.4)[a]
Spring	55	11 (20.0)[b]	27 (49.1)[b]
Summer	83	14 (16.9)[b]	42 (50.6)[b]

Values in the same column with different superscripts differ significantly (p<0.05). CPS=coagulase-positive staphylococci, CNS=coagulase-negative staphylococci.

3.3.4 Persistence of infections

CPS infections in cow quarters not cleared within 90-100 days of detection, were likely to persist throughout lactation (Fig. 3.2). The Kaplan-Meier curve indicates that less than 40% of infections were cleared within this time, and due to the number of censored cases (38/49), 90-100 days may overestimate the time required for CPS infections to become established.

Persistence of CNS isolates in cow and heifer quarters is illustrated in the Kaplan-Meier curves in Fig. 3.3. The median survival time was approximately 30 days (survival function=0.5), and 80-90% of infections were cleared within 100 days of detection.

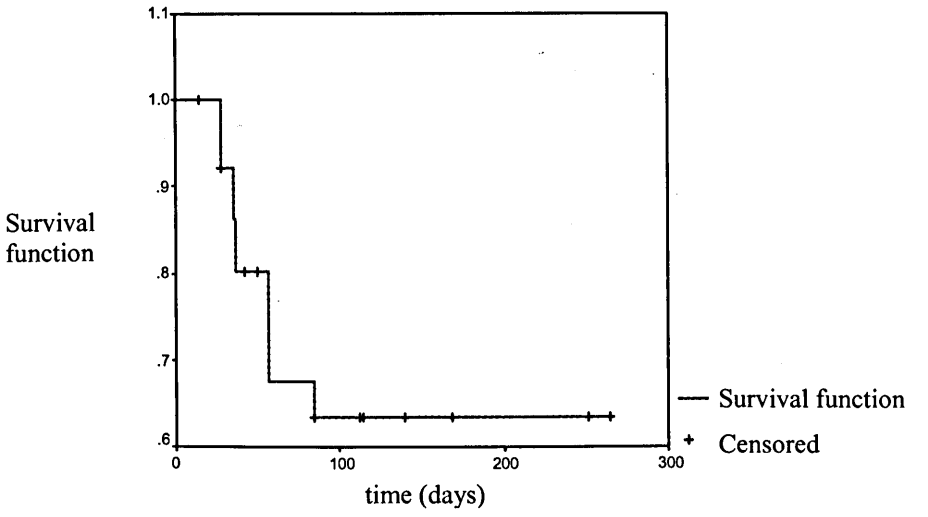


Fig. 3.2. Persistence of CPS isolates in cow quarters (n=49).

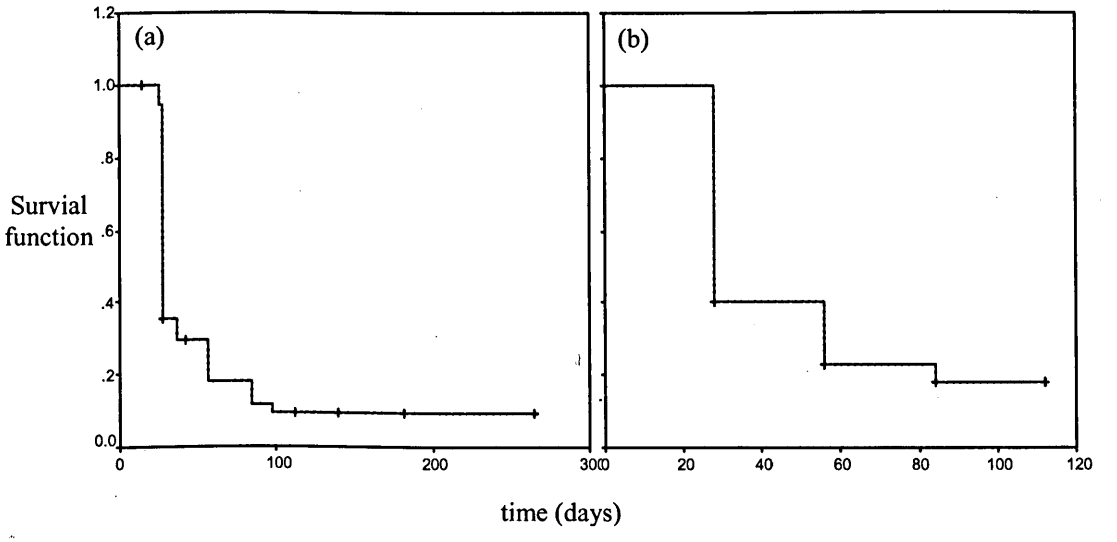


Fig. 3.3. Persistence of CNS in (a) cow quarters (n=82), and (b) heifer quarters (n=92).

— Survival function

+ Censored

Only 10% of cow quarters remained bacteriologically negative for over 200 days (Fig. 3.4a), and this may overestimate the true level due to the number of censored cases (83/128). In heifers there was a steady reduction in bacteriologically negative quarters (Fig. 3.4b). After approximately one month, 60% of quarters remained negative, but this fell to below 20% after four months. The median length of time for quarters to remain bacteriologically negative was similar for cows and heifers (50-60

days), but the shape of the curve for heifer quarters suggests a constant hazard with the risk over time remaining the same.

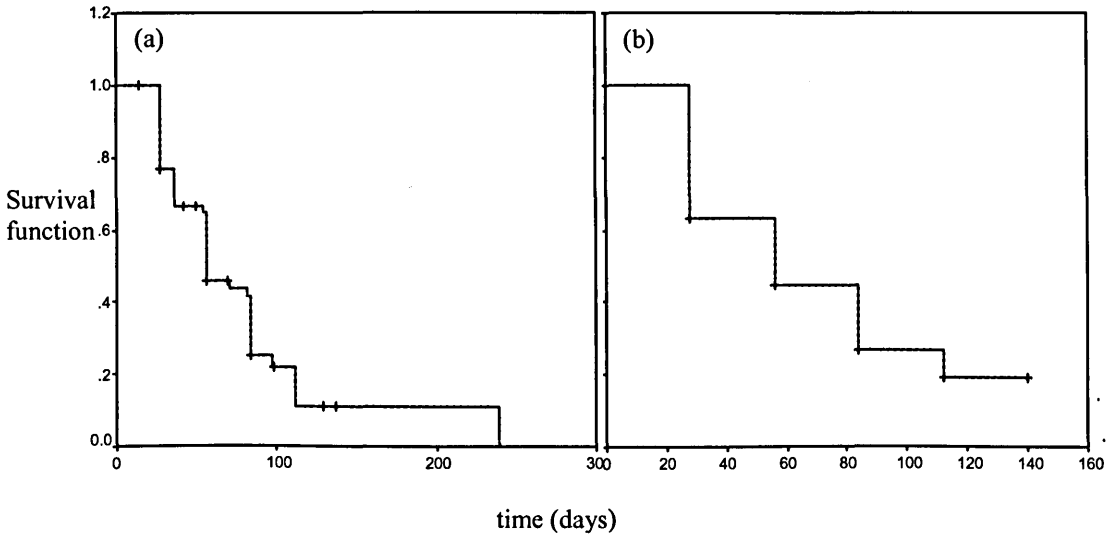


Fig. 3.4. Length of time (a) cow quarters (n=128), and (b) heifer quarters (n=99), remained bacteriologically negative.

— Survival function

\+ Censored

3.3.5 Somatic cell counts of heifers in early lactation

As cows were selected for inclusion in the study based on their SCC, this would have biased an analysis. SCC analyses therefore have been restricted to the heifers.

There was no statistically significant difference in the percentage of heifers or quarters infected with CNS or that were bacteriologically negative when the data were stratified based on individual heifer SCC (IHSCC). However an individual quarter SCC (IQSCC) below 80,000 cells ml^{-1} was significantly associated with lower levels of CNS infection (χ^2=18.64, $p<0.01$) and an increased level of bacteriologically negative quarters (χ^2=12.70, $p<0.01$) (Fig. 3.5).

The percentage of heifers with none, one, two, three or four quarters infected with CNS or bacteriologically negative did not differ significantly when they were stratified based on their IHSCC (Fig. 3.6a,b).

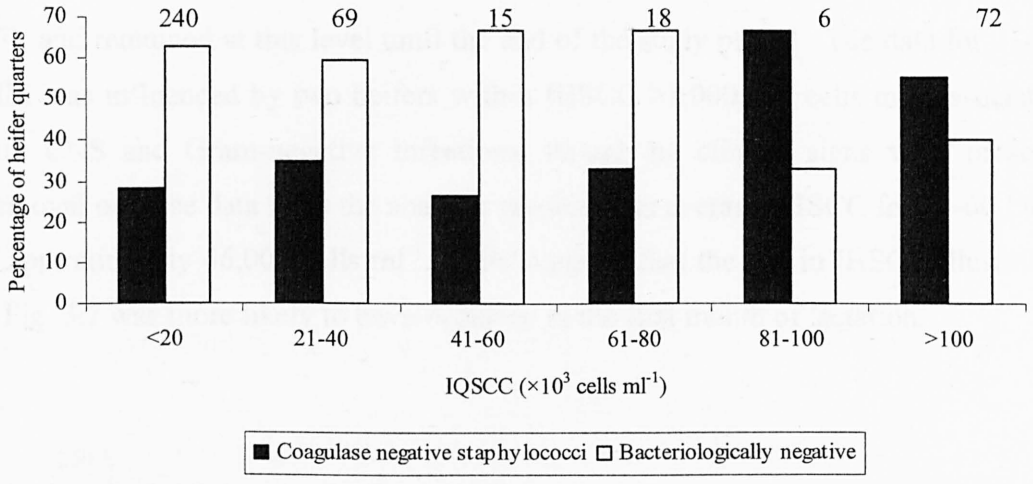


Fig. 3.5. Percentage of heifer quarters (numbers above) bacteriologically negative or infected with CNS, at a range of IQSCC.
IQSCC=individual quarter somatic cell count.

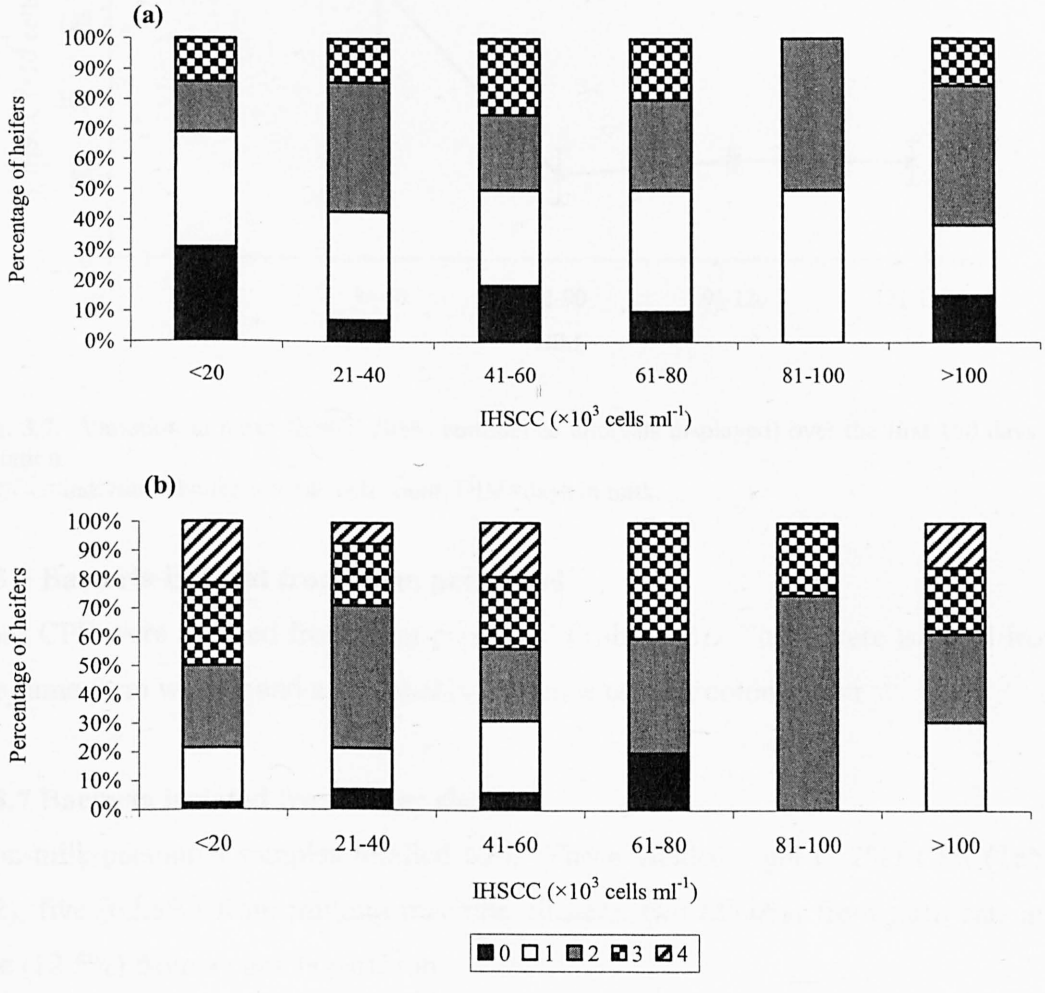


Fig. 3.6. Percentage of heifers with 0, 1, 2, 3 or 4 quarters (a) infected with CNS, or (b) bacteriologically negative.
IHSCC=individual heifer somatic cell count.

After 60 days in milk (DIM) the mean IHSCC fell from 172–51,000 cells ml^{-1} (Fig. 3.7), and remained at this level until the end of the study period. The data for 31-60 DIM was influenced by two heifers with a IHSCC >1,000,000 cells ml^{-1} associated with CNS and Gram-negative infections, though no clinical signs were present. Removal of these data from the analysis results in an average IHSCC for 31-60 DIM of approximately 46,000 cells ml^{-1}. This suggests that the fall in IHSCC illustrated in Fig. 3.7 was more likely to have occurred in the first month of lactation.

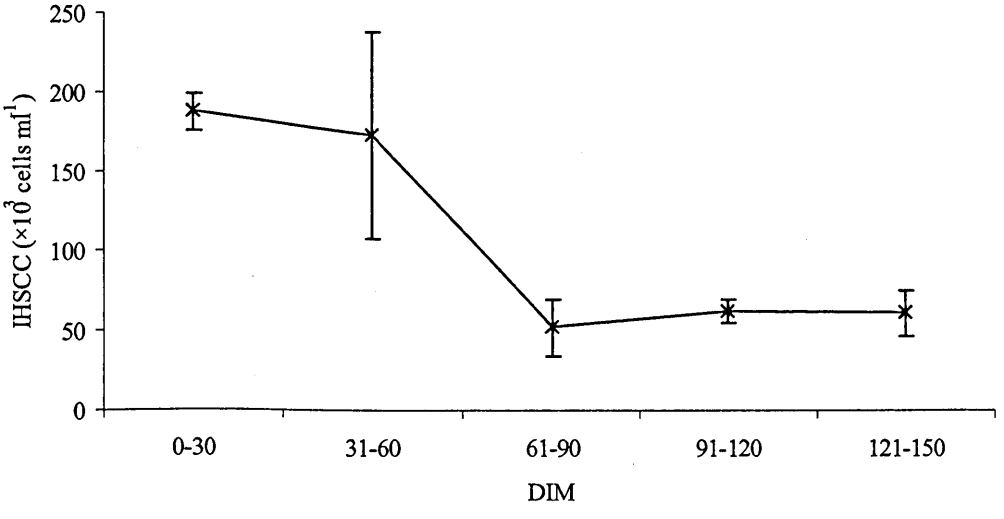


Fig. 3.7. Variation in mean IHSCC (95% confidence intervals displayed) over the first 150 days of lactation.
IHSCC=individual heifer somatic cell count, DIM=days in milk.

3.3.6 Bacteria isolated from farm personnel

Four CPS were isolated from farm personnel (Table 3.3). These were isolated from the same farm worker and are probably evidence of their colonisation.

3.3.7 Bacteria isolated from other sites

Non-milk/personnel samples totalled 676. These yielded eight (1.2%) CPS (Table 3.2), five (62.5%) from milking machine clusters, two (25.0%) from farm cats and one (12.5%) from a cubicle partition.

CNS were the most frequently isolated group of bacteria from the environmental samples, and streptococci least. However streptococci were detected in the largest

number of sites (milking machine clusters, water, environmental swabs, heifer body sites, feed and bedding).

3.4 Discussion

The aim of the work presented in this chapter was to assemble a collection of CPS from milk and a variety of environmental locations on a UK organic dairy farm for strain typing and analysis (Chapter 4). In addition, a longitudinal observational study of the infections of cows recruited into the study, and those encountered by a group of bought-in heifers entering an established organic dairy herd was conducted.

The data collected in this study were from one herd and limits the ability to generalise the results, but assembling an equivalent dataset from a large number of herds was beyond the scope of this investigation.

CPS were isolated from the milk and environment, although the small number of environmental CPS-positive sites probably indicates that these do not form a large reservoir of infection. Strain typing of the isolates detected (Chapter 4) provided more information on this aspect of study.

Monthly sampling was chosen because it was pragmatic and allowed sufficient time to process the samples and send the results to the farmer before the next visit. In contrast to recommended methods (Sears *et al.*, 1990; Buelow *et al.*, 1996*a*), diagnosis of CPS quarter infections were based on single samples as duplicate sampling would have been costly and impractical. Using monthly sampling, and diagnoses based on single samples may have affected the number of new infections, because of potential false-negative culture results. In addition the possibility also remains that some infections may have been missed altogether as they may have occurred and been cleared between visits.

The diagnostic procedures for identifying bacteria isolated from milk are well documented (Sears and Heider, 1981; Shotts and Leard, 1984; Sears *et al.*, 1993;

Hogan *et al.*, 1999; Sears and McCarthy, 2003). Culture is considered to be the 'gold standard' for diagnosis of CPS IMI (Buelow *et al.*, 1996*a*; Godden *et al.*, 2002), and has high specificity, seldom yielding false-positive results (Buelow *et al.*, 1996*b*). However the sensitivity of culture can vary (Sears *et al.*, 1990; Buelow *et al.*, 1996*a*). Pre-culture freezing of milk samples has been demonstrated to increase the number of CPS detected when they are cultured (Villanueva *et al.*, 1991; Sol *et al.*, 2002), this is particularly so for postmilking samples (Godden *et al.*, 2002), thereby improving sensitivity. Use of a large (0.1ml) volume of milk was identified by Buelow *et al.* (1996*a*) as the method that lead to the single greatest increase in sensitivity of culture methods for CPS IMI diagnosis. It was decided that use of pre-culture freezing and large inocula would be sufficiently sensitive for the purposes of this study.

Postmilking samples were collected because this reduced the likelihood of contamination as teats and teat canals are washed during milking (Sears *et al.*, 1991). Few samples were contaminated and the level [2.2%] compares favourably with the 8.3% reported by Zecconi and Piccinini (2002). The majority were detected in the first two visits, and this is probably evidence of improvement in sampling technique as the study progressed. Low levels of contamination were achieved by placing samples on-ice and returning them to the laboratory on the day of collection (Dinsmore *et al.*, 1990). Preculture incubation was not used because of time and logistical constraints, and it has been reported to increase levels of contamination (Dinsmore *et al.*, 1992). However, selective sample enrichment may have increased the recovery of CPS from environmental samples (Matos *et al.*, 1991).

Roberson *et al.* (1992) indicated that MBP agar would be suitable for the detection and differentiation of *S. aureus* from other CPS spp. However, processing of suspected *S. aureus* samples demonstrated the presence of CNS and other genera such as streptococcus spp. Further work with the CPS isolates detected in this study demonstrated that MBP agar was not successful in differentiating *S. aureus* from the other CPS spp. (Chapter 4). These results agree with the findings of Devriese (1981) and Ollis *et al.* (1995), and demonstrate that alternative techniques for differentiation of *S. aureus* from other CPS should be used, such as the PCR developed by Stepan *et al.* (2001).

The methods used for environmental CPS isolation have only succeeded in isolating CPS from certain sites when there was a high herd (>10%) prevalence of CPS IMI (Roberson *et al.*, 1994*b*). There was a CPS isolation rate of 14.8% from milk in the present study, however this is from repeated sampling of a targeted group and not the complete herd. It is likely that there was a much lower prevalence of CPS IMI on the farm, and rates of CPS isolation from non-milk sites support this view (Roberson *et al.*, 1994*b*). A conservative estimate of CPS prevalence based on the number of quarters detected with CPS at the final visit (17) and the number of cows and heifers in lactation at that time (89) is 4.8%. This level is between the cut-off values used by Roberson *et al.* (1994*b*) for distinction of high (>10%) and low (<3%) prevalence herds. There was a discrepancy in the rates of isolation from farm personnel, but this will have been biased by repeated isolation of CPS from the same farm worker.

Variation in bacterial isolation rates for individual quarters in cows is not unusual (Barkema *et al.*, 1997). Miller *et al.* (1991) reported a significant difference ($p<0.01$) in the frequency of isolation of bacteria from the four quarters, although in contrast to the current findings CPS were detected in fore quarters only. Vaarst and Enevoldsen (1997) investigating mastitis in Danish organic dairy herds also reported variation in *S. aureus* isolation rate for individual quarters. This may be explained by the lying behaviour of cattle (Ewbank, 1966). As cows tend to lie on their right side, there is an increased chance the LH teat will come into contact with the RH and possibly RF teats with the LF only likely to contact the body of the udder. This may lead to increased levels of cross contamination between rear quarters (Miller *et al.*, 1991). Statistically significantly increased levels of IMI in rear as opposed to fore quarters agrees with previous reports (Dohoo and Leslie, 1991; Miller *et al.*, 1991; Vaarst and Enevoldsen, 1997; Busato *et al.*, 2000). It has been suggested this may be because rear quarters produce more milk, or because the teats are closer to the ground, putatively exposing them to increased risk of injury (Miller *et al.*, 1991).

The statistically significantly reduced rate of CPS isolation in the LF quarter may have been influenced by the high levels of pathogens such as corynebacteria or CNS. This is also demonstrated in the LH quarter, where the lowest level of corynebacteria is coupled with the highest rate of CPS isolation. *Corynebacterium*

bovis is generally considered to be a harmless commensal (Brooks and Barnum, 1984*a*), although there is evidence for it causing clinical disease (Counter, 1981; Robinson and Harwood, 1998; Boyer, 1998). *C. bovis* has been reported to offer protection against *S. aureus* infection, although these effects are pathogen specific (Brooks and Barnum, 1984*b*; Pankey *et al.*, 1985; White *et al.*, 2001). Green *et al.* (2002) reported that the timing of infection was important, with infections occurring in the late dry/early lactation period conferring protection. It has been hypothesised that protection is most likely achieved through enhanced non-specific udder immunity (Lam *et al.*, 1997).

It was interesting to note that the rate of new CPS IMI did not vary throughout lactation. Vaarst and Enevoldsen (1997) reported that cases of CPS mastitis occurred most frequently in late lactation, and risk factors for *S. aureus* IMI are statistically significantly higher in early and late lactation (Busato *et al.*, 2000; Zecconi and Piccinini, 2002). Increased rates of CPS IMI over winter, and a fall in spring/summer may be related to housing cattle over winter. Seasonal variation in rates of clinical mastitis is not unusual (Peeler *et al.*, 2003) and it is likely that housing would increase levels of contact and mixing, putatively increasing the rate of IMI. Detection of fewer new infections in summer compared to winter/spring supports the view that hygiene improves during the grazing period (Vaarst, 2001).

There was no statistically significant variation in the rate of bacterial isolation from heifer quarters, but it was surprising that no CPS were isolated. Early and late lactation have been identified as having a statistically significantly increased risk of *S. aureus* infection, particularly for cows in their first or second lactation (Zecconi and Piccinini, 2002). The present study also differs from Matthews *et al.* (1992*a*) and Roberson *et al.* (1994*a*) who reported 7.6 and 8.1% of heifers were infected with CPS at parturition respectively.

In contrast to previous investigations (Roberson *et al.*, 1998), *S. aureus* was not detected on heifer body sites before or after parturition, indicating the main source of infection was likely to be infected mammary glands, transmission from which usually occurs at milking (Sommerhäuser *et al.*, 2003). In the present study this did not occur, and demonstrates that maintenance of herd and milking hygiene can

prevent the spread of contagious mastitis pathogens to some of the most susceptible animals, even when a substantial number of cows are infected.

Increased levels of CNS in heifers compared to cows has been described previously (Timms and Schultz, 1987; Matthews *et al.*, 1992*a*). Although in contrast to the current findings these were of long duration (Timms and Schultz, 1987). The increased rate of CNS may have afforded some protection against infection with *S. aureus* (Nickerson and Boddie, 1994). The similarity in persistence of CNS IMI in cow and heifer quarters demonstrates that while differences exist in susceptibility (Zecconi and Piccinini, 2002), once bacteria have accessed the gland, then their action (and the host's reaction) is apparently comparable. There was also similarity in rates of new IMI for cow and heifer quarters and the evidence suggests that quarters are under constant threat of infection. Thus demonstrating the requirement for good quarter (and therefore udder) management.

The low levels of Gram-negative bacteria detected were probably due to the bacteriological procedures used, as milk samples were cultured with a view to isolating Gram-positive bacteria.

No difference in crude rates of IMI with four 20,000 cells ml^{-1} IQSCC categories up to 80,000 cells ml^{-1} contrasts with the results of Peeler *et al.* (2003) who indicated that a preceeding IQSCC <20,000 cells ml^{-1} increased the risk of clinical mastitis (CM) compared to a concentration of 21-100,000 cells ml^{-1}. However the present study suffers from a small sample size comprised of cows in their first lactation, and was based on a single farm. In addition Peeler *et al.* (2003) studied clinical cases mainly caused by *Str. uberis* or coliform bacteria, and few staphylococcal infections.

The fall in IHSCC in early lactation mirrors the effects of stage of lactation on SCC described by Miller *et al.* (1991), and the residual SCC compares with the levels detected by Laevens *et al.* (1997) for cows in their first lactation. This is slightly surprising, as it is generally considered organic dairy herds have higher SCC than conventional dairy herds (Weller and Bowling, 2000). Although results of the present study may be due to investigating heifers in the first months of lactation

when there has been little opportunity for infection or a build up of somatic cells within the udder.

3.5 Conclusions and further work

The sampling and sample processing regimen employed in this study succeeded in isolating CPS from a number of sites and allowed investigation of intra-mammary bacterial infections of an organic dairy herd in the UK. Whilst the bacteriological procedures used may have underestimated the true level of infection, a number of infected quarters were detected which provided a large number of cultures for strain typing.

Environmental detection of CPS demonstrates their ability to survive outside infected mammary glands on a UK organic dairy farm. In the future however I would employ an alternative method of differentiating *S. aureus* from other CPS isolates, potentially using molecular techniques (Stepan *et al.*, 2001).

Maintenance of good udder hygiene at milking is necessary because of the constant risk of infection. The udder management skills of the dairy staff should be acknowledged, as care and attention to the milking routine and individual animals needs can only have helped to prevent the spread of contagious mastitis pathogens to the newly introduced heifers.

The variation in levels of contagious CPS by quarter merits further investigation into the effects of lying behaviour and potentially other aspects of dairy cow management on the spread of contagious mastitis pathogens. This may be of particular value to organic farmers who are forbidden from using the more conventional antibiotic based methods of controlling contagious mastitis pathogens.

Chapter 4: Multilocus sequence typing of *Staphylococcus aureus* isolated from an organic dairy farm

4.1 Introduction

Subtyping of bacteria is an important epidemiological tool (Zschöck *et al.*, 2000) and there are a number of studies investigating the number and relationships of *S. aureus* strains causing intra-mammary infections (IMI) on individual farms (amongst others: Fox *et al.*, 1991; Lam *et al.*, 1996; Lipman *et al.*, 1996; Larsen *et al.*, 2000*a*; Sommerhäuser *et al.*, 2003). These have provided important information concerning strain origin, clonal relatedness and epidemiology (Zschöck *et al.*, 2000). Many studies have detected clonal populations at the herd level, characterised by low strain diversity and the dominance of one or two strains (Lam *et al.*, 1996; Lipman *et al.*, 1996; Larsen *et al.*, 2000*a*; Zschöck *et al.*, 2000). However some studies have reported genetic diversity of *S. aureus* on individual farms (Kapur *et al.*, 1995; Joo *et al.*, 2001). These contrasting results may be a true picture of the bacterial populations on the farms studied, or they may be because different typing techniques and methods of interpretation have been used.

Recently a novel technique for the discrimination of bacteria based on the nucleotide sequences of 450-500bp internal fragments of seven house-keeping genes has been developed: multilocus sequence typing (MLST) (Maiden *et al.*, 1998; Spratt, 1999). An MLST scheme for the differentiation of *S. aureus* has been developed (Enright *et al.*, 2000), though as yet it has mainly been applied to isolates of human origin and few of animal origin (Enright *et al.*, 2000, 2002; Crisóstomo *et al.*, 2001; Day *et al*, 2002; Carter *et al.*, 2003; Feil *et al.*, 2003).

MLST offers advantages of unambiguous identification and high levels of discrimination (Spratt, 1999) because it is based on specific gene sequences at seven loci. It is suitable for global (Urwin and Maiden, 2003) and local (Peacock *et al.*, 2002) epidemiology and permits world-wide inter-laboratory comparisons via the internet (http://www.mlst.net). In addition MLST data are amenable to studies investigating population structure and evolutionary analyses of nucleotide sequence data (Feil *et al.*, 2003; Urwin and Maiden, 2003).

The aim of the work presented in this chapter was to determine whether MLST is suitable for the differentiation of *S. aureus* isolated from a UK organic dairy farm (Chapter 3). Further aims were to investigate relationships between the strains, determine the effect (if any) of strain variation on pathogenesis and investigate the persistence of strains within mammary quarters.

This is the first occasion MLST has been applied to large numbers of *S. aureus* isolated from bovine mammary secretions, and the first investigation into the number and distribution of *S. aureus* strains on an organic dairy farm.

4.2 Materials and methods

4.2.1 Source of bacterial isolates

The isolates analysed in this chapter were from a UK organic dairy farm (Chapter 3). One hundred and forty two milk samples containing coagulase-positive staphylococci (CPS), and 12 non-milk associated CPS isolates were available for multilocus sequence typing (MLST).

4.2.2 Multilocus sequence typing

4.2.2.1 DNA isolation protocols

Initially phenol:chloroform DNA isolation was employed, however this was labour intensive, produced poor quality DNA and had low sample throughput (20 samples/day). To overcome these problems the Qiagen DNeasy® Tissue Kit (69504; Qiagen, Crawley, UK) and DNeasy® 96 Tissue Kit (69581; Qiagen) were used. These produced much higher quality DNA, and had a greater sample throughput (up to 192 samples/day). The protocols followed are described below:

Phenol:chloroform DNA isolation protocol: Bacterial stocks (stored at –80°C) were streaked across sheep's blood agar (SBA; Oxoid, Basingstoke, UK) containing 5% sheep's blood (TCS Biosciences Ltd., Buckingham, UK) to culture isolated colonies. One colony was picked off using a swab (SS 359; Appleton Woods, Selly Oak, UK),

spread across the surface of another 5% SBA (Oxoid) plate and incubated at 37°C overnight to create a lawn of confluent bacterial culture.

Half the culture was harvested using a swab (Appleton Woods) moistened in lysis solution 1 (Table 4.1) and resuspended in 400µl of lysis solution 1 in a sterile 1.5ml microfuge tube. Samples were incubated at 37°C for 30min followed by 95°C for 10min, placed on ice and 1ml of phenol:chloroform:isoamyl alcohol (25:24:1; C-0549; Sigma-Aldrich Co. Ltd, Poole UK) was added and mixed by inversion. Samples were centrifuged at 16,060×*g* for 20min and the aqueous layer recovered to a fresh sterile 1.5ml microfuge tube. DNA was precipitated by adding 1ml ice-cold (-20°C) ethanol, and the samples placed on ice for 15min. The samples were centrifuged, as before, for 20min, the liquid removed, and the pellet dried in a vacuum. The pellet was resuspended in 50µl sterile water with 1µl RNase DNase free (1 119 915; Roche Diagnostics). To test for the presence of DNA, samples were run by electrophoresis on a 1% (w/v) ultraPure agarose (Gibco BRL, Paisley, UK) gel [1g agarose in 100ml 1×TAE buffer] containing 0.025µl ml^{-1} ethidium bromide (E-2515; Sigma-Aldrich). The gel was visualised and photographed under UV illumination (UVP Grab-It software, UVP, Upland, CA). DNA samples were stored at –20°C.

Table 4.1. Lysis solutions used in DNA isolation protocols.

	Lysis solution 1 (ml)	Lysis solution 2 (ml)
5000Uml^{-1} lysozyme (0.1mg ml^{-1})	0.5	0.5
500U ml^{-1} lysostaphin (0.25 mg ml^{-1})	0.5	0.5
0.5M EDTA (pH 8.0)	0.2	0.2
1.0M Tris-HCl (pH 7.5)	0.1	0.1
12% Triton x-100		1.0
Sterile water	8.7	7.7

DNeasy® and DNeasy® 96 Tissue Kit DNA isolation protocols: Bacterial stocks were streaked out, single colonies inoculated into 10ml sterile tryptone soya broth (TSB) (Oxoid) and incubated at 37°C overnight. Five hundred microlitres of overnight culture was aliquoted into a sterile 1.5ml microfuge tube and bacteria pelleted by centrifugation at 5,000×*g* for 5min. Bacteria were resuspended in 180µl lysis solution 2 (Table 4.1) (based on the recommended lysis solution (DNeasy® Tissue Kit Handbook, 05/2002) and Killgore *et al.*, 2000).

Following resuspension, the bacteria were incubated at 37°C for 1h. Twenty-five microlitres of proteinase K (supplied) and 200µl of kit buffer AL (DNeasy® Tissue Kit) or kit buffer AL/E (no ethanol added) (DNeasy® 96 Tissue Kit) were added, the samples mixed by vortexing and then incubated at 70°C for 30min. Two hundred microlitres of 100% ethanol were added and the samples mixed by vortexing before being centrifuged at 1,500×*g* for 10s to remove any droplets from the inside of the lids. Six hundred and fifteen microlitre aliquots were transferred to the individual DNeasy® spin columns (DNeasy® Tissue Kit) or the separate columns in the DNeasy® 96 plate and sealed with an air pore tape sheet (DNeasy® 96 Tissue Kit).

From this point, the protocols followed the manufacturers recommendations, but were modified by warming the kit buffer AE to 37°C (DNeasy® Tissue Kit) and by performing all centrifugations at 2,250×*g* for 15min (DNeasy® 96 Tissue Kit). Flow-through (eluted DNA) was treated with 1µl (500µg ml^{-1}) RNase DNase-free (Roche Diagnostics) and incubated at 37°C for 30min.

All samples were run on a 1% (w/v) ultraPure agarose (Gibco BRL) gel containing 0.025µl ml^{-1} ethidium bromide (Sigma-Aldrich), visualised and photographed under UV illumination (UVP) to test for the presence of DNA. Isolated DNA was stored at -20°C and diluted 1:10 with sterile water for use in PCR reactions.

4.2.2.2 PCR protocols

The primers (Table 4.2) and protocols (Table 4.3) developed by Stepan *et al.* (2001) targeting a fragment of DNA partially encoding ORF 1 and ORF 2, were used to confirm isolates were *S. aureus*, before analysing with the MLST primers (Table 4.2) developed by Enright *et al.* (2000).

PCRs were prepared using Maxymum Recovery barrier tips (Axygen Scientific Inc., Union City, CA) and carried out in 0.2ml thin-walled PCR tubes (AB-0620; ABgene, Epsom, UK) or 96-well plates (AB-0900; ABgene) in a final reaction volume of 50µl. This contained 5µl 10× PCR buffer (supplied with the *Taq* DNA polymerase), 1.5mM $MgCl_2$, 0.2mM each of dATP, dCTP, dGTP and dTTP (10297-018; Invitrogen Ltd., Paisley, UK; and R0181; MBI Fermentas, Helena Biosciences Ltd., Tyne & Wear, UK), 100ng of forward and reverse primer (Gibco BRL), 1U of *Taq*

DNA polymerase (Gibco BRL and EP0402; MBI Fermentas) and 5µl of chromosomal DNA preparation. Thermal cycling was carried out in Hybaid MBS 0.2S and 0.2G PCR machines (Thermo Hybaid, Ashford, UK) under the control of the MultiBlock System (MBS) software (MBS v.1.08 Hybaid). All reactions had a 5min initial denaturation stage before cycling, and a 5min final extension step. Annealing temperatures and cycling conditions for all primers were optimised (Table 4.3).

Table 4.2. PCR primer sequences.

Target/gene	Primer	Sequence (5'-3')	Amplicon size (bp)
ORF 1/ORF 2 (partial)	JIRS-2	AAA AAC ACT TGT CGA TAT GG	
	JIRS-1	GTT TCA ATA CAT CAA CTC C	826
Carbamate kinase	*arcC*-Up	TTG ATT CAC CAG CGC GTA TTG TC	
(*arcC*)	*arcC*-Dn	AGG TAT CTG CTT CAA TCA GCG	456
Shikimate	*aroE*-Up	ATC GGA AAT CCT ATT TCA CAT TC	
dehydrogenase (*aroE*)	*aroE*-Dn	GGT GTT GTA TTA ATA ACG ATA TC	456
Glycerol kinase (*glpF*)	*glpF*-Up	CTA GGA ACT GCA ATC TTA ATC C	
	glpF-Dn	TGG TAA AAT CGC ATG TCC AAT TC	465
Guanylate kinase	*gmk*-Up	ATC GTT TTA TCG GGA CCA TC	
(*gmk*)	*gmk*-Dn	TCA TTA ACT ACA ACG TAA TCG TA	429
Phosphate	*pta*-Up	GTT AAA ATC GTA TTA CCT GAA GG	
acetyltransferase (*pta*)	*pta*-Dn	GAC CCT TTT GTT GAA AAG CTT AA	474
Triosephosphate	*tpi*-Up	TCG TTC ATT CTG AAC GTC GTG AA	
isomerase (*tpi*)	*tpi*-Dn	TTT GCA CCT TCT AAC AAT TGT AC	402
Acetyl coenzyme A	*yqiL*-Up	CAG CAT ACA GGA CAC CTA TTG GC	
acetyltransferase (*yqiL*)	*yqiL*-Dn	CGT TGA GGA ATC GAT ACT GGA AC	516

bp=base pairs

To check the PCRs had successfully amplified DNA, 5µl of product was mixed with 2µl loading buffer (Gel loading buffer type III; Sambrook *et al.*, 1989) and run on a 1% (w/v) ultraPure agarose (Gibco BRL) gel containing 0.025µl ml^{-1} ethidium bromide (Sigma-Aldrich). Samples were visualised and photographed under UV illumination (UVP). Two or three products from high throughput MLST PCR reactions (96-well plate format), were run on a gel to ensure successful amplification of the target gene. Products were stored at 4°C (overnight) or at –20°C (for longer periods) until they were purified and prepared for sequencing.

4.2.2.3 PCR product purification

PCR products were purified using Qiagen MinElute™ 96 UF PCR Purification Kits (28051 and 28053; Qiagen) in concert with the QIAvac Multiwell unit (9014579; Qiagen) fitted to a vacuum pump as recommended by the manufacturer (MinElute™ 96 UF PCR Purification Handbook, 05/2002).

Table 4.3. PCR conditions used for the amplification of DNA.

Primer	Denaturation		Annealing		Extension		Cycles
	Temp. (°C)	Time (min)	Temp. (°C)	Time (min)	Temp. (°C)	Time (min)	
JIRS	94	1	44	2	74	1	40
arcC	95	1	55	1	72	1	35
aroE	95	1	55	1	72	1	30
glpF	95	1	55	1	72	1	30
gmk	95	1	51	1	72	1	40
pta	95	1	55	1	72	1	35
tpi	95	1	51	1	72	1	40
yqiL	95	1	55	1	72	1	30

Temp.=temperature

4.2.2.4 Gene sequencing

DNA concentration in the purified PCR products was determined by comparison of band intensities. Two microlitres of sample was mixed with 2µl loading buffer and run alongside a low DNA molecular mass ladder (10068-013; Invitrogen) in a 1% (w/v) ultraPure agarose (Gibco BRL) gel containing 0.025µl ml^{-1} ethidium bromide (Sigma-Aldrich). One hundred to one hundred and twenty nanograms of DNA and 3.2pmol of sequencing primer (same sequence as PCR primers) was then suspended in a final volume of 6µl, and submitted to the Molecular Biology Service, University of Warwick for sequencing as described below. Individual samples were prepared and submitted in 0.5ml microfuge tubes, high throughput samples were prepared in 96-well plates (THER-FA7; Elkay, Basingstoke, UK) and sealed with adhesive PCR film (AB-0558; ABgene).

Cycle sequencing: Individual samples were transferred to 96-well plates by the Molecular Biology Service. The 6µl sample was cycled in a total volume of 20µl containing 4µl BigDye Terminator (version 3.1) Ready Reaction Cycle Sequencing Kit (Applied Biosystems (ABI), Warrington, UK) and 2µl 5× dilution buffer (supplied). For *gmk* cycle sequencing 1µl DMSO (D/4121/PB08; Fisher Scientific UK, Loughborough, UK) was also included to improve sequence quality and reduce secondary structures. Cycling was carried out on a GeneAmp PCR System 9700 (ABI) machine with initial denaturation at 96°C for 5min, followed by 25 cycles of denaturation at 96°C for 10s, annealing at 50°C for 5s and extension at 60°C for 4min. Once completed, samples were held at 4°C.

Unincorporated dye terminators and any remaining salts, proteins and detergents were removed by ethanol precipitation. Two microlitres of 0.125mM EDTA (E 7889; Sigma-Aldrich Co. Ltd., Poole, UK), 2µl 3M NaOAC (pH 5.2) (S-7899; Sigma-Aldrich) and 50µl 96% (v/v) ethanol were added and vortexed. Samples were incubated at room temperature for 20min and centrifuged at 4°C, 2,250×*g* for 30min. The supernatant was discarded and the samples centrifuged inverted (4°C, 50×*g*, 1min) before 150µl 70% (v/v) ethanol was added to each well and mixed by vortexing. The plate was again centrifuged (4°C, 2,250×*g*, 20min), the supernatant discarded, followed by another brief inverted centrifugation and the plates were then stored dry at –20°C.

Prior to being run through the sequencer, samples were resuspended in 12µl of HiDi formamide (ABI), denatured at 95°C for 2min and centrifuged at 4°C, 2,250×*g* for 1min to remove any air bubbles. Samples were then run on the ABI Prism Genetic Analyser 3100 (ABI) and their sequence determined as described below.

The capillaries (16×, 50cm long) in the sequencer were purchased ready-filled with a proprietary acrylamide mix (performance optimised polymer 6; POP6). DNA was separated through the polymer in the capillaries and the order of bases recorded as they passed through a laser beam. Raw data were collected from the laser using a charge-coupled device (CCD) camera and ABI Prism 3100 Genetic Analyser Data Collection Software version 1.1 (ABI). Results were edited and corrected for dye mobilities in ABI Prism Sequencing Analysis Software version 3.7 (ABI) to give a final output in .ab1 (ABI) file format.

4.2.2.5 Sequence analysis

Sequences were returned as .ab1 (ABI) trace files. Forward and reverse traces (*viewed in*: Chromas v. 1.45, Conor McCarthy, Griffith University, Australia) of one locus from one isolate were saved as text files (EditSeq; DNAStar 5, DNASTAR Inc., Madison, WI), imported into SeqMan (DNAStar) and aligned alongside a reference sequence for that locus (downloaded from http://www.mlst.net). Sequences were trimmed to the correct length (as determined by the reference sequence) so they corresponded to the region used to define the alleles. The

consensus sequence i.e. that determined by the forward and reverse sequences (which may differ from the reference sequence), was saved as a text file.

Allele and Sequence Type assignment: Consensus sequences were compared with the sequences in the database via the online form, the software checked sequences were the correct length and did not contain unrecognised characters (http://www.mlst.net). If a match was not found, this was likely to be a novel allele at that locus. In this case forward and reverse .abl (ABI) trace files of the putative new allele were sent to the *S. aureus* database curator (M.C. Enright, University of Bath) for approval, allele number assignment and entry into the database.

Once all seven alleles had been assigned for each isolate, this provided the isolate's allelic profile (AP) which was used to determine it's sequence type (ST), again via the website (http://www.mlst.net). For novel STs, the APs of the strains were sent to the database curator for ST assignment and entry into the database.

4.2.3 Data storage and analysis

Data generated were entered into a Microsoft (MS) Excel (Excel 97: Microsoft Corp.) spreadsheet for analysis. Statistical analysis of the data was performed using Chi-Square Tables (Epi Info version 6.04d; Centers for Disease Control & Prevention (CDC), Atlanta, GA).

Frequency of polymorphic sites was determined in SeqMan (DNAStar) by aligning sequences of interest and identifying conflicts. Synonymous and non-synonymous amino acid changes were detected by translating genetic sequences into protein-coding sequences in EditSeq (DNAStar), aligning the new sequences in SeqMan (DNAStar) and highlighting any conflicts.

Strain persistence was investigated using Kaplan-Meier survival curves. These were constructed in SPSS (SPSS 10.0 Client/Server, Release 10.0.5.336: SPSS Inc., Chicago ILL) after adjusting the data generated in Chapter 3 to take into account strain variation. Data were stored in MS Excel (Microsoft Ltd.), analysed and presented in SPSS (SPSS). Curves were presented with survival function on the Y-

axis, a value of one was equal to 100% infection survival, survival function of 0.5 (50%) indicated the median time to recovery from infection.

Phylogenetic analyses were conducted using Molecular Evolutionary Genetics Analysis (*MEGA*) version 2.1 (Kumar *et al.*, 2001). The seven allele sequences were concatenated in the order *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, *yqiL* to produce a 3198bp sequence for each ST. These were stored as text files in *MEGA* format and used as the dataset for analysis. The Neighbour-Joining method of dendrogram construction was used, and bootstrap sampling was carried out to estimate the reliability of the dendrogram.

MLST was evaluated by determining the typeability, discriminatory power and reproducibility for the isolates under study, as recommended by Struelens *et al.* (1996*a*,*b*). The diversity of the isolates based on MLST was calculated using the equation for determining Simpson's index of diversity (D) (Hunter and Gaston, 1988), with 95% confidence intervals (CI) calculated using the equations of Grundmann *et al.* (2001). Five isolates were selected at random and duplicate samples processed to determine the reproducibility of MLST when applied to *S. aureus* of bovine origin.

4.2.4 Case definitions

Infection: The detection of *S. aureus* in the milk sample collected.

Persistent infection: The same sequence type isolated from the same quarter at successive visits.

Recovered/Cleared: If *S. aureus* had been detected at one visit and was not isolated from the same quarter at the following visit.

Censored: Data collection ended before the infection cleared, e.g. due to culling, drying-off or no further samples were taken.

4.3 Results

The 142 milk samples containing CPS yielded 183 CPS isolates selected by haemolytic variation. Using the JIRS PCR primers, 182 (99.5%) were confirmed as *S. aureus*, and one (0.5%) as a CPS other than *S. aureus* (most likely *S. hyicus* or *S. intermedius*) (Table 4.4). Of the 12 non-milk associated CPS, three (25.0%) were CPS other than *S. aureus*. A total of 191 isolates were analysed by MLST.

4.3.1 Performance of MLST

The typeability of MLST was 100%, i.e. all isolates were successfully typed. The discriminatory power (D) was 0.590 (95% confidence intervals=0.547-0.633), indicating that if two isolates were selected at random from the population, on 59.0% of occasions they would be identified as different STs. The five duplicate samples analysed to determine reproducibility of MLST yielded the same result as the initial sample, giving a reproducibility of 100%.

Table 4.4. Number (%) and distribution of confirmed *S. aureus* CPS, and sequence types.

	Site					Total
	Milk	Clusters	Personnel	Environment	Non-bovines	
CPS	183	5	4	1	2	195
S. aureus	182 (99.5)	4 (80.0)	4 (100.0)	1 (100.0)	0 (0.0)	191 (97.9)
ST25	7 (3.9)	1 (25.0)	0	0	0	8 (4.2)
ST30	0	0	4 (100.0)	0	0	4 (2.1)
ST116	69 (37.9)	1 (25.0)	0	0	0	70 (36.7)
ST117	4 (2.2)	0	0	0	0	4 (2.1)
ST118	94 (51.6)	2 (50.0)	0	1 (100.0)	0	97 (50.8)
ST119	2 (1.1)	0	0	0	0	2 (1.0)
ST127	6 (3.3)	0	0	0	0	6 (3.1)

Non-bovines=non-bovine animals, CPS=coagulase-positive staphylococci, ST=sequence type.

4.3.2 Sequence types isolated from milk

The 182 milk-associated *S. aureus* consisted of six STs (ST25, ST116, ST117, ST118, ST119 and ST127; Table 4.4). ST116 and ST118 made up 89.5% of the milk isolates and 87.5% of the total collection. ST25, ST127, ST117 and ST119 formed 3.9, 3.3, 2.2 and 1.1% of the milk isolates respectively and were often isolated in association with one other ST. Of the six STs isolated from milk, ST118, ST25 and ST116 were also isolated from the milking machine clusters forming 50, 25 and 25% of the cluster isolates respectively. Only ST118 was isolated from the farm environment, and ST30 was isolated from farm personnel (Table 4.4).

Two new alleles at the *tpi* locus (*tpi*59 and *tpi*60), and five new STs (ST116, ST117, ST118, ST119 and ST127) were discovered (Table 4.5), and have been added to the *S. aureus* MLST database (http://www.mlst.net).

Table 4.5. Sequence types and allele numbers detected.

Sequence Type	Allele numbers						
	arcC	*aroE*	*glpF*	*gmk*	*pta*	*tpi*	*yqiL*
25	4	1	4	1	5	5	4
30	2	2	2	2	6	3	2
116[1]	3	1	1	1	1	59[2]	3
117[1]	3	1	1	1	5	59[2]	4
118[1]	3	1	1	1	1	60[2]	3
119[1]	3	1	1	1	5	60[2]	4
127[1]	4	1	4	1	1	5	3

[1]novel sequence types detected
[2]novel alleles detected

Sequence Type interactions: ST116 and ST118 were detected in the same quarter once (Cow 6, visit 6; Table 4.6), although neither infection persisted. At the quarter level the two seemed to be mutually exclusive, although at the animal level three cows (Cows 13, 37 and 44) were persistently infected with both strains in different quarters. On one occasion (Cow 65) ST116 was replaced with ST118 (Table 4.6) although the detected infection did not persist.

ST25 and ST127 were detected in Cow 34 in separate quarters (RH and RF respectively) before she was dried off at the end of her fourth lactation (between visit 2-3). ST127 was detected in the same quarter (RF) at the first sampling of her next lactation, but ST25 was not. The RF quarter remained persistently infected, however at visit 8 (80 days in milk) the strain detected was ST25, not ST127 and it was ST25 that persisted until the end of the study. ST127 was not isolated again, and none of Cow 34's other quarters became infected with a detectable level of *S. aureus*.

ST117 was isolated from Cows 2 and 1 at the first and second visits respectively. The LH quarter of cow 2 was infected with ST117 at the first visit, and with ST116 at the second; the opposite was true for the LF quarter of Cow 1. After the second visit these cows were culled. On the other occasion ST117 was detected, it was in association with ST116 (Cow 5, visit 7, RF). A high level (>1000 cfu ml^{-1}) of both

isolates were detected (data not shown), and this quarter was persistently infected with ST116 throughout the study period (Table 4.6).

Table 4.6. STs detected in cows and quarters by visit.

Cow ID	Qrt	Visit number (monthly)									
		1	2	3	4	5	6	7	8	9	10
1	LF	116	117	Cull							
1	LH	118	118	Cull							
1	RH	118	118	Cull							
2	LH	117	116	Cull							
2	RH	118	NSD	Cull							
4	LF	NSD	116	Cull							
4	LH	116	116	Cull							
4	RF	116	116	Cull							
4	RH	116	116	Cull							
5	LH	116	116	116	116	116	116	116	116	116	Dry
5	RF	116	116	116	116	116	116	116/7	116	116	Dry
5	RH	NSD	NSD	NSD	NSD	NSD	116	NSD	116	116	Dry
6	RH	NSD	NSD	Dry	Dry	Dry	116/8	NSD	NSD	NSD	NSD
8	RH	-	-	-	-	-	-	118	-	-	-
11	RF	NSD	Dry	Dry	NSD	NSD	NSD	NSD	NSD	NSD	118
13	LH	-	-	-	-	118	118	118	118/9	118	118
13	RF	-	-	-	-	116	116	116	116	116	116
13	RH	-	-	-	-	118	118	NSD	NSD	NSD	NSD
16	LF	-	-	-	-	-	-	118	118	118	118
16	LH	-	-	-	-	-	-	118	118	118	NSD
16	RH	-	-	-	-	-	-	118	118	118	118
20	LF	-	-	-	-	-	-	-	-	118	118
20	LH	-	-	-	-	-	-	-	-	118	118
20	RF	-	-	-	-	-	-	-	-	118	118
20	RH	-	-	-	-	-	-	-	-	118	118
22	LF	NSD	118	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD
22	LH	NSD	118	NSD	NSD	118	118	118	118	118	118
22	RF	118/9	118	118	118	118	118	118	118	118	118
22	RH	118	118	118	118	118	118	118	118	118	118
32	LH	118	Dry	Dry	Dry	Dry	Dry	Dry	Dry	Dry	Cull
32	RH	NSD	Dry	Dry	Dry	NSD	118	NSD	NSD	NSD	Cull
34	RF	127	NSD	Dry	Dry	Dry	127	127	25	25	25
34	RH	NSD	25	Dry	Dry	Dry	NSD	NSD	NSD	NSD	NSD
37	LH	116	Dry	Dry	Dry	116	116	116	116	116	116
37	RH	NSD	Dry	Dry	Dry	NSD	NSD	118	118	118	118
44	LH	116	Dry	Dry	116	116	116	116	116	116	116
44	RH	118	Dry	Dry	118	118	NSD	NSD	NSD	NSD	NSD
46	LH	116	Dry	Dry	NSD	NSD	NSD	NSD	NSD	NSD	NSD
64	LF	-	-	-	-	-	-	118	-	-	-
65	LH	116	Dry	Dry	Dry	NSD	NSD	116	118	NSD	NSD
71	RF	-	-	-	-	-	-	118	118	118	118

25	ST25 detected
116	ST116 detected
117	ST117 detected
118	ST118 detected
127	ST127 detected
116/8	Mixed infection detected (in this case ST116 and ST118)
NSD	No *S. aureus* detected
Dry	Cow in her dry period
Cull	Cow culled
-	No sample taken

Qrt=quarter
LF=left fore
LH=left hind
RF=right fore
RH=right hind

ST119 was detected twice, both times in association with ST118 (Table 4.6), ST118 was the dominant strain in terms of the number of cfu ml^{-1} detected (data not shown).

In the following analyses ST116 and ST118 have been considered in detail, however where appropriate the detection of STs with few data points has been presented.

Somatic cell counts: At low individual cow SCC (ICSCC) (<100,000 cells ml^{-1}) few *S. aureus* were detected (Fig. 4.1). However, ST25 and ST127 were only isolated at an ICSCC of below 201,000 cells ml^{-1}. The proportion of quarters infected with ST116 remained steady in cows with an ICSCC >100,000 cells ml^{-1}, although within the 401-500,000 cells ml^{-1} range it increased, but this may be due to low sample numbers. In contrast the proportion of quarters infected with ST118 increased at higher ICSCC with statistically significantly more ($p<0.01$) ST118 detected than ST116 in cows with an ICSCC of 301-400 and >501,000 cells ml^{-1} ($\chi^2=10.30$ and 7.78 respectively).

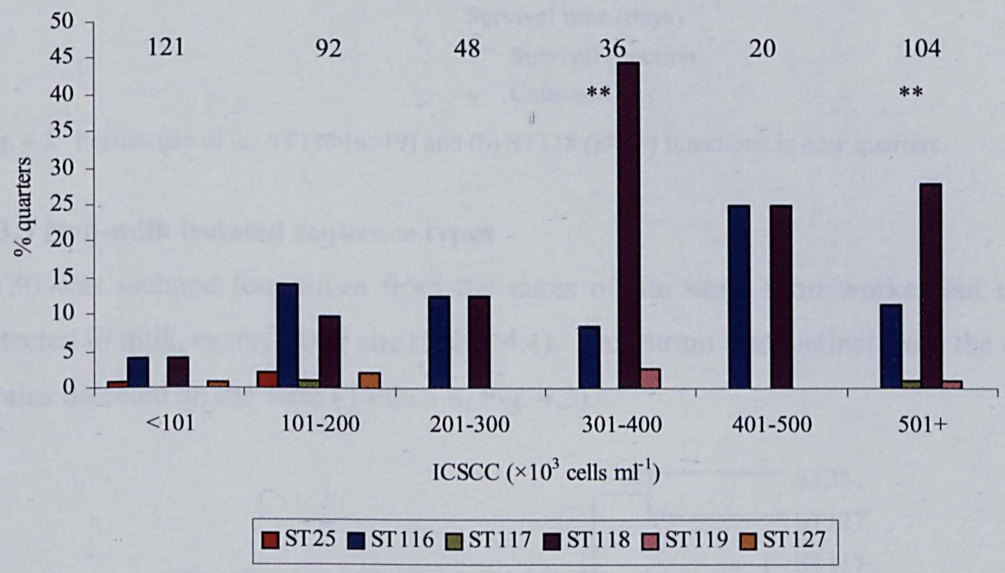


Fig. 4.1. Number of quarters sampled and percent infected with each sequence type, by ICSCC. **Significant difference in the proportions of ST116 and ST118 ($p<0.01$), ICSCC=individual cow somatic cell count.

Survival analyses: The persistence of ST116 and ST118 (Fig. 4.2) within mammary quarters is presented in the Kaplan-Meier curves in Fig. 4.2. Less than 50% of infections were cleared, and the optimum time for removal of infection was within 50 (ST116) or 100 (ST118) days of detection. Beyond this, infections persisted for

over 250 days (probably to the end of lactation). The mean survival time for ST116 and ST118 in cow quarters was 183 (95% confidence intervals (CI)=128-238) and 158 (95% CI=107-210) days respectively. However some of the data used to produce these figures were censored e.g. by culling infected animals. This will have biased the results as infection with *S. aureus* was taken in to consideration as a reason for culling and reduced the length of time a minimum of five (ST116) and two (ST118) (Table 4.6) infections could be investigated.

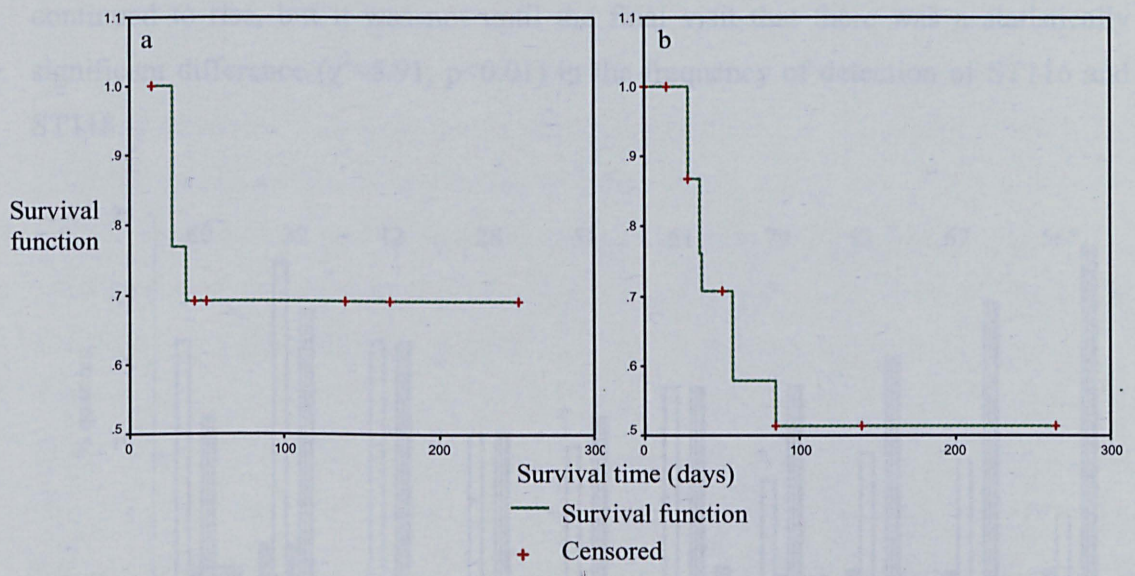


Fig. 4.2. Persistence of (a) ST116 (n=19) and (b) ST118 (n=28) infections in cow quarters.

4.3.3 Non-milk isolated sequence types

ST30 was isolated four times from the nares of the same farm worker but never detected in milk, or any other site (Table 4.4). This strain was distinct from the other strains detected on the farm (Table 4.6, Fig. 4.3).

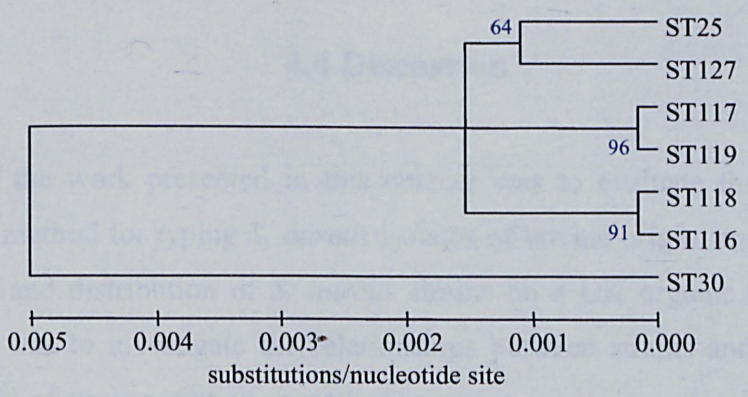


Fig. 4.3. Dendrogram of the seven sequence types detected.

4.3.4 Organic farm *S. aureus* population

The variation in the proportion of quarters infected with each ST by visit is illustrated in Fig. 4.4. The fall in numbers of infected quarters at visits 3 and 4 was because many of the cows enrolled in the study were in their dry period. As cows returned to lactation, the rate of detection of quarters infected with the two dominant STs remained steady until visit seven when an increase in sample numbers collected (due to a number of cows with a high SCC) led to a greater proportion of ST118 being detected. From this point on the proportion of quarters infected with ST118 continued to rise, but it was not until the final visit that there was a statistically significant difference (χ^2=5.91, p<0.01) in the frequency of detection of ST116 and ST118.

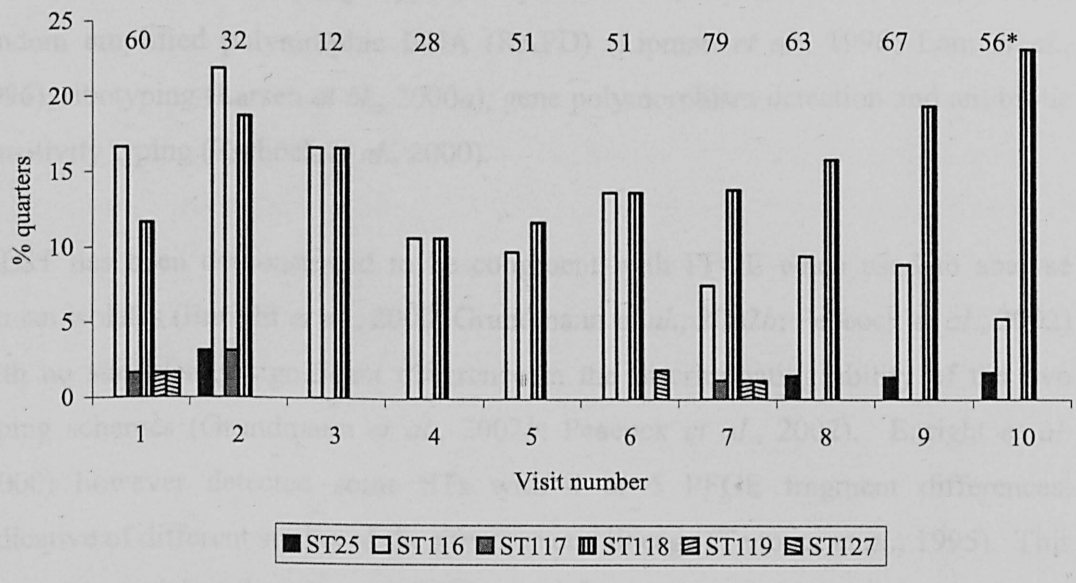


Fig. 4.4. Number (%) of quarters infected with each sequence type, by visit.
*proportions of ST116 and ST118 differed significantly (p<0.01).

4.4 Discussion

The aim of the work presented in this chapter was to evaluate the usefulness of MLST as a method for typing *S. aureus* isolates of bovine origin, and to investigate the number and distribution of *S. aureus* strains on a UK organic dairy farm. A further aim was to investigate the relationships between strains and determine the effect (if any) of strain variation on pathogenicity.

MLST has high (100%) typeability and reproducibility when applied to bovine *S. aureus*. This is the same level of typeability as PFGE, and compares favourably to phage and binary typing (Zadoks *et al.*, 2002). Previous work with human isolates has demonstrated that large numbers of human strains of *S. aureus* are also typeable using MLST (Enright *et al.*, 2000; Grundmann *et al.*, 2002*b*).

The high discriminatory power of MLST has revealed a low level of diversity on the farm in the present study. This is likely to be due to the clonal population structure of *S. aureus* (Lam *et al.*, 1996; Lipman *et al.*, 1996; Larsen *et al.*, 2000*a* and Zschöck *et al.*, 2000; Feil *et al.*, 2003). At the herd level, MLST has a comparable level of discriminating ability as techniques such as pulsed-field gel electrophoresis (PFGE) (Zschöck *et al.*, 2000), phage typing (Lipman *et al.*, 1996; Larsen *et al.*, 2000*a*) random amplified polymorphic DNA (RAPD) (Lipman *et al.*, 1996; Lam *et al.*, 1996), ribotyping (Larsen *et al.*, 2000*a*), gene polymorphism detection and antibiotic sensitivity typing (Zschöck *et al.*, 2000).

MLST has been demonstrated to be congruent with PFGE when used to analyse human isolates (Enright *et al.*, 2000; Grundmann *et al.*, 2002*b*; Peacock *et al.*, 2002) with no statistically significant difference in the discriminating ability of the two typing schemes (Grundmann *et al.*, 2002*b*; Peacock *et al.*, 2002). Enright *et al.* (2000) however detected some STs with 4 or 5 PFGE fragment differences, indicative of different strains of the same genetic lineage (Tenover *et al.*, 1995). This was not surprising though, as MLST assesses variation accumulated slowly and PFGE assesses variation accumulated relatively rapidly (Maiden *et al.*, 1998).

However, MLST is more expensive and time-consuming than other molecular techniques, although increasing use of automation will help to reduce these costs. It may be more prudent in similar studies in the future to initially perform a preliminary screening of isolates e.g. using RAPD (Lipman *et al.*, 1996; Lam *et al.*, 1996) or fluorescent amplified-fragment length polymorphism (FAFLP; Grady *et al.*, 1999). The results could then be used to select the dominant strains (or all, if only a limited number are detected) for MLST analysis and comparison at a wider level.

Detection of a limited number of strains with variation at one or two loci, and the dominance of one or two strains is indicative of a clonal population (Enright and Spratt, 1999; Spratt and Maiden, 1999), and strain distribution may be related to virulence, demonstrated by the ability to persist within the host (Schlegelová *et al.*, 2003). This supports the theory of cow to cow transmission (Zschöck *et al.*, 2000) although the environment was also identified as a putative reservoir of infection.

The identification of novel alleles is probably evidence of the herd/farm/cow selection pressures creating localised variants of *S. aureus* (Matthews *et al.*, 1994; Su *et al.*, 1999; Buzzola *et al.*, 2001), and is not unusual for MLST analyses of animal isolates (Carter *et al.*, 2003). The presence (at the time of writing) of no strains from other locations in the *S. aureus* MLST database with alleles *tpi*59 and *tpi*60 (http://www.mlst.net) supports the theory of localised strain evolution. The non-synonymous changes detected may have improved the ability of *S. aureus* to persist and cause disease following invasion of the mammary gland. Indeed a bovine-mastitis associated *S. aureus* clone (RF122), has recently been demonstrated to have elevated rates of non-synonymous substitution compared to the human-associated clones Mu50 and N315, which may be related to host specificity (Herron *et al.*, 2002).

The change from a population with two dominant strains to a situation where one dominated the other is similar to the change in prevalence of genotypes detected by Buzzola *et al.* (2001) over a number of years in Argentina. However this is probably not a true picture of the farm population as the sampling targeted high ICSCC cows [ICSCC >400,000 cells ml^{-1} for two consecutive months]. Towards the end of the sampling period, more cows were enrolled in the study because of high ICSCC, and there is evidence to support the theory of strain specific pathogenicity causing a variation in SCC response. This is demonstrated by ST25 and ST127 which were never isolated from a cow with an ICSCC above 200,000 cells ml^{-1} and the statistically significant difference in the isolation rate of ST116 and ST118 from cows with a high (>501,000 cells ml^{-1}) ICSCC. The low detection rate of ST25/ST127 was probably because of targeting high SCC cows. It may be that cows with a lower ICSCC are colonised with different strains of *S. aureus*, indicating a difference in strain-specific virulence. This would go some way to explaining

incidences of *S. aureus* mastitis in herds with low SCC (Torgerson *et al.*, 1992). Although a low ICSCC response to a *S. aureus* infection may also be due to stage of infection and dilution effects of milk from uninfected quarters (Hoblet *et al.*, 1988).

The hypothesis of strain dependent pathogenicity is supported by Middleton and Fox (2002) who detected statistically significant differences in levels of milk production between quarters infected with 'Novel strain *S. aureus*' and uninfected control quarters; and no difference between quarters infected with 'other *S. aureus* strains' and uninfected control quarters. Zadoks *et al.* (2000) also observed a significant correlation between *S. aureus* strain type and disease characteristics.

The reduced SCC response induced by some strains of *S. aureus* may be due to variation in plasmid carriage (Piccinini and Zecconi, 2001). A 2027bp plasmid has been identified which seemingly has a higher probability of allowing it's carrying strain to invade and colonise mammary glands, resist antimicrobial therapy and remain undetected (Piccinini and Zecconi, 2001). The potential for strains to avoid detection is supported by the increased mean survival time of ST116 compared to ST118 in mammary quarters. This was probably because statistically significantly more ST118 caused a higher ICSCC response than ST116, potentially leading to the conclusion that cows infected with ST118 were 'more infected' than cows with ST116. Though in practice, these effects will be blurred if a cow is infected with both strains.

Caution should be taken in the wider application of these principles as most studies are based on few, or geographically restricted isolates, and the present investigation is no exception. In contrast to these results though, Middleton *et al.* (2002) could find no difference in the pathogenesis of different *S. aureus* strains, but suggested that there was a large cow to cow variation in response to IMI with a given strain of *S. aureus*. However from an initial prevalence study which identified 411 infected cows, only 191 were studied. If farmers were informed of the bacteriology results, they may have culled or dried off the high SCC cows, leaving the animals with a lower SCC response for further study. Whilst this is unlikely to have removed all 'high SCC causing' strains from each herd, it may well have biased the sample population.

The persistence of *S. aureus* infections and the relatively short period following initial detection when infections were cleared agrees with previous work indicating early detection and treatment is optimal for removal of *S. aureus* infections (Hillerton *et al.*, 1995). However the bias induced by culling infected animals probably means that the optimum time for removal of infection is less than indicated.

The data suggests that the STs detected can be split into pairs (ST116/ST117, ST118/ST119 and ST25/ST127) which are able to co-exist within cow quarters. The same loci varied for each pair, and for two pairs (ST116/ST117 and ST118/ST119) allele numbers 1 and 3 at the *pta* and *yqiL* loci dominate respectively, for the other (ST25/ST127) the opposite seems to be true. However, all the data for the latter pair originated from one cow, and this will have biased the results. At the *pta* locus the variation between the alleles resulted in three polymorphic sites and two non-synonymous changes, at the *yqiL* locus there were four polymorphic sites and one non-synonymous change. Detection of more than one strain of *S. aureus* in individual quarters has been demonstrated previously (Young *et al.*, 2001), although this was an experimental as opposed to natural infection. Similar findings have also been demonstrated in cases of *Str. uberis* mastitis (Jayarao *et al.*, 1991). Persistence of dual strain infections in the same quarter was not detected, but this may be due to the lack of colonies investigated (one, or two if there was haemolytic variation). More routine analysis of multiple colonies may provide a better understanding of *S. aureus* IMI epidemiology (Young *et al.*, 2001). Though it could be that bacterial population dynamics within quarters promote the predominance of one strain. This theory is supported by Young *et al.* (2001) who were unable to establish non-indigenous *S. aureus* in a naturally infected quarter, and hypothesised that this may be due to competition for physiological or pathological requirements.

The intra-quarter ability of *S. aureus* strains to dominate was not reflected at the inter-quarter level, with the dominant strains able to infect different quarters of the same cow. This has been demonstrated previously (Zadoks *et al.*, 2000), and for cases of *E. coli* (Bradley and Green, 2001) and *Str. uberis* mastitis (Jayarao *et al.*, 1991; Wieliczko *et al.*, 2002).

Sporadic detection of more than one strain within a quarter may be evidence for an environmental style *S. aureus* as postulated by Zadoks (2002), who suggested that heterogeneity and low frequency of isolation may be explained by environmental origin. Examples of such strains in the present study are ST117 and ST119, and from other studies of farms with overall clonal *S. aureus* populations include those with DNA restriction patterns II and V (Annemüller *et al.*, 1999), ribotypes 6, 11 and 57 (Larsen *et al.*, 2000*a*), isolates 6 and 7 (Zadoks *et al.*, 2000) and $R_{2,3,4,6,7}$ (Young *et al.*, 2001). Thus the potential for such environmental strains of *S. aureus* is not uncommon. Unfortunately ST117/ST119 were not isolated from environmental samples in this study, though this may be due to limitations in the sampling and processing protocols.

The repeated detection of *S. aureus* from the nares of a farm worker is probably evidence of their persistent infection. The ST detected (ST30) has been identified as the major methicillin-susceptible *S. aureus* (MSSA) clone associated with invasive disease in the Oxford region (Enright *et al.* 2000). Determination of this strain as different from those detected in cows agrees with previous reports that strains isolated from farm workers differ from those detected in cattle (Fox *et al.*, 1991; Lopes *et al.*, 1990; Kapur *et al.* 1995; Larsen *et al.*, 2000*b*; Zadoks *et al.*, 2000; and Schlegelová *et al.* 2003), and that they are typical of the human reservoir present in the country of detection (Larsen *et al.*, 2000*a*). The variation in strains isolated from different sites is discussed in more detail in Chapter 5.

In Chapter 3, MBP agar was used to differentiate *S. aureus* from other CPS, and based on this, all CPS were classed as *S. aureus*. However use of the JIRS PCR primers developed by Stepan *et al.* (2001) to confirm this result prior to MLST indicated that one (0.5%) of the CPS detected in milk and four (30.8%) non-milk associated isolates were CPS other than *S. aureus*. The identity of these isolates was not determined, but it demonstrates that CPS other than *S. aureus* are culturable on MBP agar. This also demonstrates a low prevalence of CPS other than *S. aureus* in milk, and is lower than the 2-30% detected in cows by Roberson *et al.* (1996) and the 3% reported by Capurro *et al.* (1999). However these authors were investigating the prevalence over a number of herds, not sampling cows longitudinally. Detection of CPS other than *S. aureus* in the farm environment demonstrates that they too are able

to survive outside mammary glands. It may be that some of the CPS other than *S. aureus* were able to invade mammary glands (particularly as some were detected in milking machine clusters) and not lead to enough of an increase in ICSCC to cause concern, this merits further investigation.

4.5 Conclusions and further work

MLST is a useful and highly discriminatory molecular typing technique suitable for typing isolates of *S. aureus* from bovine mammary secretions, however at the farm (herd) level it would probably be more efficient to use an alternative technique such as FAFLP for differentiating *S. aureus*. Further analysis of not only the basic genetic make-up of isolates but other factors such as antibiotic sensitivity profiles, presence/absence of toxins and/or plasmid carriage of isolates will provide much greater indication of the epidemiology of *S. aureus* isolated from IMI. It would be wise to perform these analyses with well-established techniques to allow comparison of strains from a number of distinct sources. MLST could then be reserved for typing the most common strains (or all if few are detected) on individual farms, for comparison with others collected and typed at national and global levels.

Future strain-typing investigations into the epidemiology of *S. aureus* (and possibly all causes of) bovine mastitis would benefit from the analysis of multiple colonies from each milk sample. This will give a greater overview of the true patterns of bacterial populations causing IMI and determine whether certain strains predominate. In addition it may generate data as evidence for strain combinations within mammary quarters, and whether certain strain combinations are more likely to occur. If this is the case, the relative proportions of the strains and investigations of their virulence determinants such as plasmid profiles and antibiotic susceptibility patterns would provide interesting data about IMI patterns. This however is unlikely, at least in the immediate future as the costs incurred in identifying numerous colonies per milk sample to the strain level would be prohibitive.

Use of MLST has demonstrated that there is significant genetic variation of *S. aureus* even on one farm, and it is probable that localised selection pressures led to unique *S. aureus* variants. This means that the number of sites/samples and level of discrimination (typing method) have to be chosen based on specific questions, e.g. a project investigating rates of transmission ('strain following') would have different requirements to one studying herd level populations.

The potential for different strains to cause differences in the host immune response, putatively leading to undetected reservoirs in seemingly uninfected animals is interesting, and warrants further investigation. There may also be a case for investigation of CPS other than *S. aureus* in cows.

The sporadic detection of some strains adds support to the theory of environmental *S. aureus* on dairy farms.

The reservoir of *S. aureus* detected in the farm staff differed to that detected in the cows and was probably not important with respect to causing IMI, though further analysis of these strains would provide more conclusive results.

The use of MBP agar for the identification of *S. aureus* proved not to be specific and should not be used as a method for differentiation of CPS. It would be more accurate to use molecular techniques such as the PCR developed by Stepan *et al.* (2001).

Chapter 5: Multilocus sequence typing of bovine *Staphylococcus aureus* from the USA and Chile

5.1 Introduction

Mastitis is the biggest problem facing dairy farmers worldwide, and *S. aureus* is a major cause. Traditionally *S. aureus* has been considered a contagious pathogen i.e. is spread from cow to cow probably via the milking machine (Newbould, 1968; Zschöck *et al.*, 2000). However environmental strains of *S. aureus* have been isolated from dairy farms (Fox *et al.*, 1991; Matos *et al.*, 1991; Roberson *et al.*, 1994*b*; Chapter 3).

A number of studies have investigated strain variation of *S. aureus* isolated from dairy farms (Fox *et al.*, 1991; Roberson *et al.*, 1998; Buzzola *et al.*, 2001; Joo *et al.*, 2001; Zadoks *et al.*, 2002) to pinpoint potential sources and routes of spread. In addition a number of studies have examined the relationship between *S. aureus* isolated from bovine mastitis from different countries/continents (Kapur *et al.*, 1995; Fitzgerald *et al.*, 1997, 2000; Su *et al.*, 1999, 2000). Studies investigating the global population structure of bovine *S. aureus* suggest there are relatively few, specialised clones responsible for the majority of IMI (Fitzgerald *et al.*, 1997; Kapur *et al.*, 1995). Although investigations of virulence factors such as coagulase gene polymorphism (Su *et al.*, 1999) and toxin production (Fitzgerald *et al.*, 2000) indicated that genotypes differ among geographic locations, with few prevailing at each site (Su *et al.*, 1999).

Most studies have used techniques such as phage typing (Fox *et al.*, 1991; Roberson *et al.*, 1998) and PFGE (Buzzola *et al.*, 2001; Joo *et al.*, 2001) to compare isolates. These methods lack inter-centre reproduceability (Zadoks *et al.*, 2002). Library typing systems such as binary typing (van Leeuwen *et al.*, 1998) and multilocus sequence typing (MLST) (Maiden *et al.*, 1998) have been developed to overcome these problems by producing results that are repeatable between laboratories and over time.

The aim of the work presented in this chapter was to investigate the effectiveness of MLST as a method for typing *S. aureus* of bovine origin from a number of distinct geographical sources, and to compare it to PFGE typing. A further aim was to examine a collection of isolates previously characterised using phage (Fox *et al.*, 1991), PFGE and binary typing (Zadoks *et al.*, 2002). The MLST data were then to be used in a preliminary analysis of the evolutionary and population biology of *S. aureus* of bovine origin.

5.2 Materials and methods

5.2.1 Bacterial isolates

Two hundred and thirty-one epidemiologically well-defined isolates of *S. aureus*, from milk (117), teat skin (TS; 75), milking machine unit liners (34), milkers' hands (four) and dairy cow bedding (one) (*courtesy of*: Dr. L. K. Fox and Prof. Y. H. Schukken) were typed. The strain collection has been described elsewhere (Fox *et al.*, 1991; *identified hereafter as*: the 'Fox collection'). Briefly, *S. aureus* was isolated from composite milk samples collected from 43 herds sampled once during the second year of a management survey (Corbeil *et al.*, 1984). Herds were considered for inclusion if *S. aureus* IMI and/or teat skin colonisation had been detected (Fox *et al.*, 1991).

A further 32 isolates of *S. aureus* were typed. These were isolated from milk submitted by private veterinary practitioners to the University of Austral, Chile (20; *courtesy of*: Drs. J. Kruze and N. Tadich), and 11 from IMI of cows in Somerset (*courtesy of*: Dr. A. J. Bradley, University of Bristol). The Chilean isolates were from 14 communes, of four provinces of two regions in the South of Chile, and so were probably representative of bovine associated *S. aureus* from these regions. In addition, the reference strain *S. aureus* Newbould 305 (NCIMB 702892) originally isolated from the milk of a cow with non-severe mastitis (Prasad and Newbould, 1968) was typed. This gave a total of 263 *S. aureus* isolates to be typed using MLST.

5.2.2 Multilocus Sequence Typing

For a full description of the methods used see section 4.2.1.

Duplicate samples of three isolates selected at random were typed to investigate the reproducibility of MLST.

5.2.3 Data storage and analysis

Data storage and analysis was performed as described in section 4.2.2, in addition to the methods described below. The ST detected from the UK (Chapter 4) were included in the analyses where indicated. Methods used for the population structure analysis have been used to investigate the population structure of *S. aureus* isolated from humans (Feil *et al.*, 2003).

Statistical analysis of the data was performed using Chi-Square tables (Epi Info version 6.04d, CDC).

Comparison of typing techniques: PFGE typing results of the Fox collection (Zadoks *et al.*, 2002) were available (*courtesy of*: Prof. Y. H. Schukken) for comparison. There were 205 isolates with corresponding MLST profiles. Results were compared using cross-tabulation (Microsoft).

Sequence type interactions: Relationships between the proportions of STs detected per herd and site of isolation for the Fox collection were determined using cross tabulations (Microsoft).

Assignment to clonal complexes: The program BURST (Based Upon Related Sequence Types) was used to divide the isolates into clonal complexes (Enright *et al.*, 2002). A clonal complex was defined as a group of STs which share five of seven alleles with at least one other ST in the group. The ST that gave rise to the clonal group (the ancestral ST) diversified initially at one of the seven loci to produce single locus variants (SLVs), therefore ancestral STs were assigned on the basis that they defined the most SLVs. The pattern of descent of each ST from the ancestral ST is displayed in a series of concentric circles. The central (solid) circle

contains the ancestral ST, the SLVs in the next (solid) circle, and double locus variants (DLVs) in the outer (dashed) circle. Single and double locus variants of STs other than the ancestral ST were connected by a solid (SLV) or dashed (DLV) line.

The enhanced version of BURST (eBURST; Feil *et al.*, 2004) was used to examine all MLST data produced, alongside the entire *S. aureus* MLST database (http://www.mlst.net). This gave an indication of how a clonal complex may have emerged and diversified. Initially data were divided into groups of STs which shared identical alleles at six of the seven loci. The primary founder of each group was assigned on the same principles as the BURST ancestral ST, and therefore is equivalent to the BURST ancestral ST. Assignment did not take into account number of isolates, to avoid sampling bias. eBURST used lines to demonstrate radial links from the founder to SLVs, and as only SLV links were shown, DLVs of the founder were only displayed if the intermediate SLV was present in the input data. On the eBURST diagram the circle representing the primary founder was coloured blue and the areas of each circle indicated the prevalence of each ST in the input data. A bootstrapping procedure was used to provide the level of confidence in assigning the primary founder.

Phylogenetic analysis: For each ST the seven locus sequences were concatenated in the order *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi* and *yqiL* to produce a 3,198bp sequence. These were stored as text files in *MEGA* (Molecular Evolutionary Genetics Analysis) format and used as the dataset for analysis. Splits graphs (Dress *et al.*, 1996; Huson, 1998) were constructed by producing a matrix of pairwise differences for the STs in *MEGA* v.2.1 (Kumar *et al.*, 2001). The matrix was pasted into the text area for split-decomposition analysis on the MLST website (http://www.mlst.net), and analysed using Splitstree v.3.2 (Dress *et al.*, 1996; Huson, 1998).

Dendrograms were constructed for individual loci and the concatenated sequences in *MEGA* v2.1 (Kumar *et al.*, 2001) using the Neighbour-Joining method, bootstrap sampling was carried out to estimate reliability.

Estimates of recombination: The methods of Feil *et al.* (2003) were used to estimate recombination. Briefly, SLV STs identified by BURST were compared to the

ancestral ST to determine the variant locus. The ancestral and variant locus were compared in the data explorer in *MEGA* v.2.1 (Kumar *et al.*, 2001) to determine nucleotide changes; sequences were translated into amino acid sequences and compared to identify synonymous and non-synonymous changes. The ratio of non-synonymous to synonymous substitutions (d_N/d_S) was calculated using the START (Sequence Type Analysis and Recombinational Tests) software package version 1.0.5 (Jolley *et al.*, 2001), available from http://outbreak.ceid.ox.ac.uk/software.htm. Allele changes by point mutation were assigned to the alleles that differed at one nucleotide site, and produced alleles which were unique to that ST; alleles not satisfying these criteria were assigned as having arisen by recombination. d_N/d_S ratios >1 implied selection for amino acid change, and < 0.035 indicated the genes were highly conserved (Dingle *et al.*, 2001; Herron *et al.*, 2002)

5.3 Results

5.3.1 Performance of MLST and comparison with PFGE

A total of 259 *S. aureus* isolates were typed by MLST, this consisted of 227 typeable isolates from the Fox collection (three were spp. other than *S. aureus*, one was non-typeable), 20 isolates from Chile, 11 from the UK and the reference strain *S. aureus* Newbould 305 (NCIMB 702892).

The typeability of MLST for the Fox collection was 99.6% (Struelens *et al.*, 1996*a*,*b*), and the discriminatory power (D) was 0.74 (95% confidence intervals (CI)=0.61-0.87; Hunter and Gaston, 1988; Grundmann *et al.*, 2001). The isolates processed in duplicate yielded the same results for both analyses, giving a reproducibility of 100%.

MLST and PFGE results were available for 12 STs (Fig. 5.1). Four were represented by more than one PFGE type (Table 5.1). Of these, ST124 was not statistically significantly associated with a single PFGE type and was split into 5 PFGE types, three of which (C, G and H) made up 89.0% of the isolates. None of these PFGE types (C, G and H) were statistically significantly associated with a particular herd

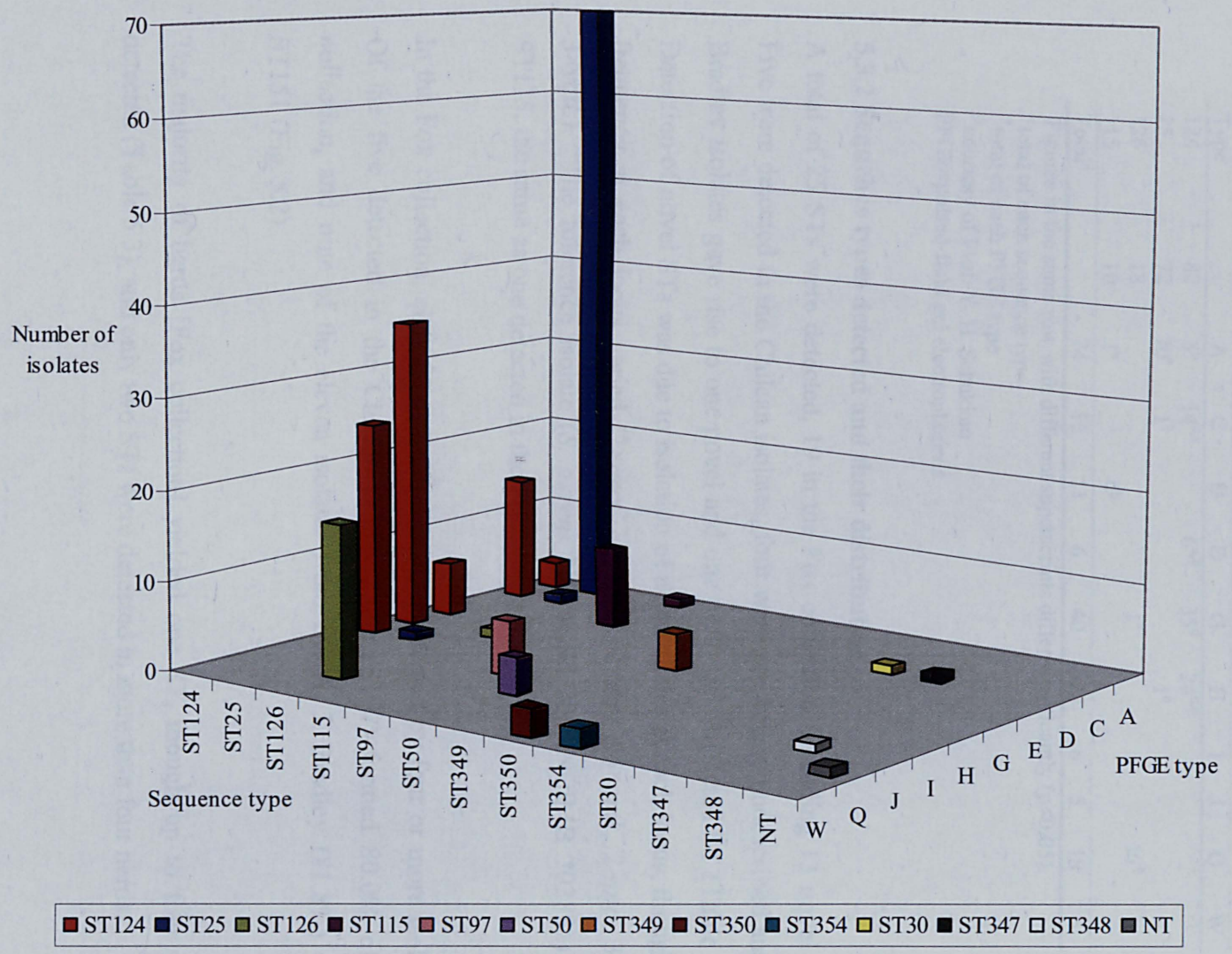


Fig. 5.1. Comparison of MLST and PFGE typing results of the Fox collection.
NT=non-typeable, PFGE=pulsed-field gel electrophoresis.

and there was no statistically significant difference ($p>0.05$) in the isolation rates of ST124 or any of the PFGE types from milk (data not shown).

Table 5.1. Sequence types divided into more than one PFGE type.

Sequence Type	Total[1]	PFGE type[3]									
		A	C	D	E	G	H	I	J	Q	W
124	82	3^{a}	$14^{b,c}$		$6^{a,b}$	35^{d}	$24^{c,d}$				
25	72	70^{a}	1^{b}				1^{b}				
126	18					1^{a}				17^{b}	
115	10	1^{a}		9^{b}							
Total[2]		74	15	11	6	40	25	6	5	18	5

Figures in the same row with different superscripts differ significantly ($p<0.05$).
[1]total of each sequence type
[2]total of each PFGE type
[3]courtesy of Prof. Y. H. Schukken
PFGE=pulsed-field gel electrophoresis

5.3.2 Sequence types detected and their distribution

A total of 25 STs were detected, 19 in the Fox collection, including 13 novel STs. Five were detected in the Chilean isolates, four were previously undescribed and the Bradley isolates gave rise to one novel and one previously detected ST (Table 5.2). Detection of novel STs was due to isolation of novel alleles at each locus, though the frequency at each locus varied (2×*arcC*, 7×*aroE*, 2×*glpF*, 4×*gmk*, 2×*pta*, 3×*tpi*, 3×*yqiL*). The reference isolate (*S. aureus* Newbould 305; NCIMB 702892) was ST115, the same as one detected in the Fox collection.

In the Fox collection, eight STs (93.0%) were represented by four or more isolates. Of the five detected in the Chilean samples, one (ST97) formed 80.0% of the collection, and nine of the eleven isolates from Dr. A. J. Bradley (81.8%) were ST151 (Fig. 5.2).

The majority of herds [Fox collection] yielded one ST, though up to four were detected (Table 5.3), and only two STs were detected in more than four herds.

Table 5.2. Sequence types and allele numbers detected from international bovine *S. aureus* isolates.

Isolate source	Sequence Type (ST)	Allele numbers detected						
		arcC	*aroE*	*glpF*	*gmk*	*pta*	*tpi*	*yqiL*
USA	1	1	1	1	1	1	1	1
USA	25	4	1	4	1	5	5	4
USA	30	2	2	2	2	6	3	2
USA	45	10	14	8	6	10	3	2
USA	50	16	16	12	2	13	13	15
USA	97	3	1	1	1	1	5	3
USA	115[1]	3	1	1	1	1	5	53[2]
USA	122[1]	27	14	8	6	10	3	2
USA	124[1]	3	1	1	37[2]	1	5	3
USA	126[1]	3	68[2]	1	4	1	5	40
USA	285[1]	2	2	2	4	6	3	2
USA	347[1]	3	1	1	37[2]	1	5	53[2]
USA	348[1]	16	16	12	4	13	13	15
USA	349[1]	1	1	1	37[2]	1	5	53[2]
USA	350[1]	6	79[2]	51[2]	47[2]	7	70[2]	61[2]
USA	351[1]	6	72[2]	50[2]	43[2]	52[2]	67[2]	59[2]
USA	352[1]	3	78[2]	1	1	1	5	3
USA	353[1]	8	2	2	46[2]	2	2	2
USA	354[1]	6	79[2]	51[2]	47[2]	53[2]	70[2]	61[2]
Chile	97	3	1	1	1	1	5	3
Chile	355[1]	49[2]	1	1	1	1	5	3
Chile	356[1]	48[2]	80[2]	50[2]	43[2]	49	71[2]	59[2]
Chile	357[1]	3	82[2]	1	1	1	5	62[2]
Chile	358[1]	3	81[2]	1	1	1	5	3
UK	9	3	3	1	1	1	1	10
UK	151[1]	6	72[2]	12	43[2]	49	67[2]	59[2]
NCIMB	115[1]	3	1	1	1	1	5	53[2]

NCIMB=National collection of industrial and marine bacteria
[1]novel sequence types detected
[2]novel alleles detected

Table 5.3. Number of sequence types detected per herd.

ST per herd	No. (%) of herds
1	25 (58.1)
2	11 (25.6)
3	5 (11.6)
4	2 (4.7)

ST=sequence type.

5.3.3 Sequence type and site of isolation interactions

Among the ST represented by four or more isolates, ST50 (n=4) and ST349 (n=4) were only isolated from milk, whilst ST115 (n=13) was never detected in milk (Fig. 5.3). There was a statistically significant difference ($p<0.05$) in the isolation frequency of ST25, ST115, ST124 and ST126 from TS and milk.

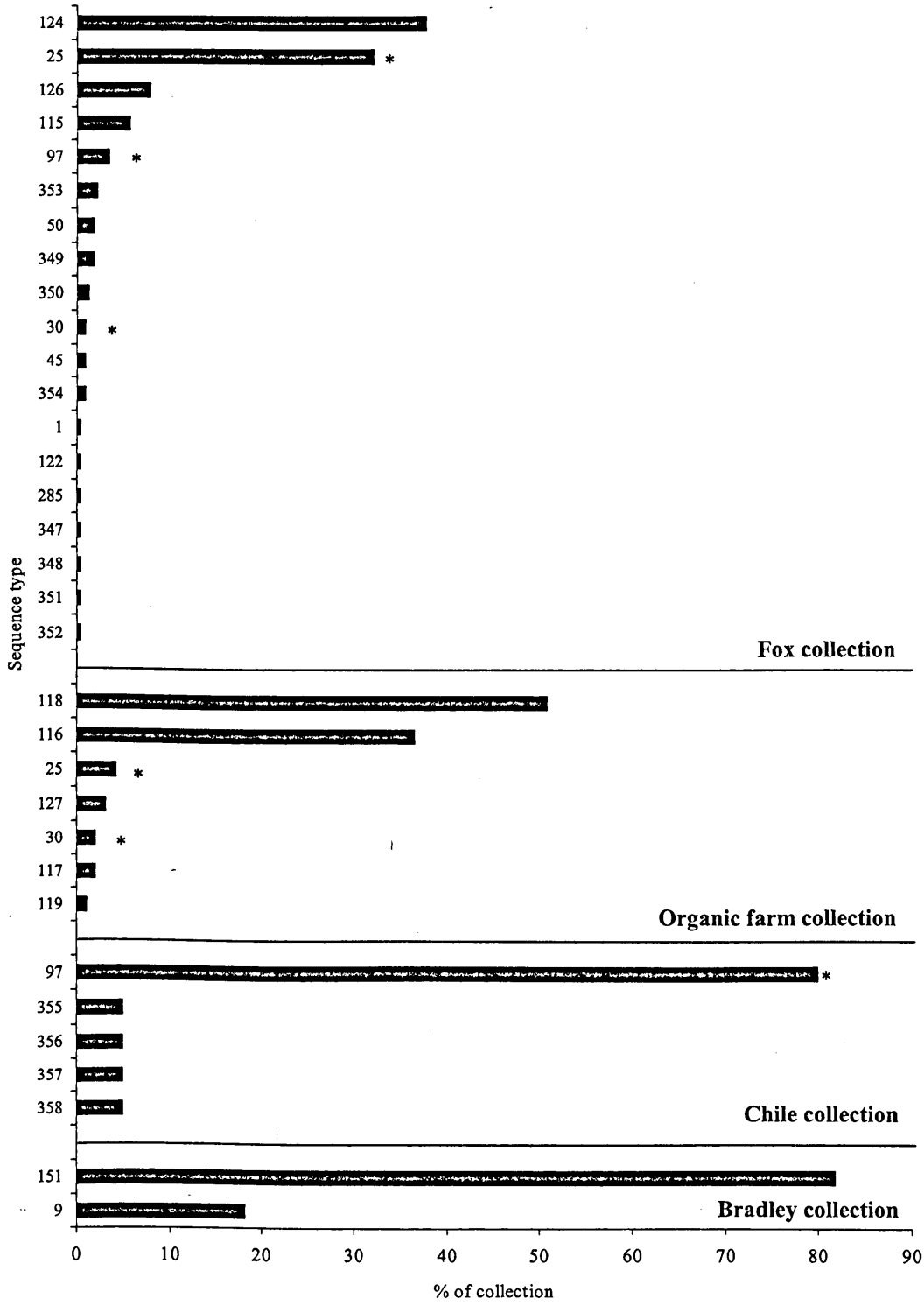


Fig. 5.2. Percentage of bovine *S. aureus* isolates from each country, by sequence type.
*sequence types detected in more than one collection.

There was a greater likelihood on individual farms [Fox collection] of detecting the same ST on teat skin and liners, than there was from milk and liners, and milk and

teat skin (Table 5.4). However, these differences were not statistically significant ($p>0.05$). In four herds, *S. aureus* was isolated from milkers hands, of these, three had a positive milk sample but only one the same ST. However all four herds gave a positive TS sample and three yielded the same ST.

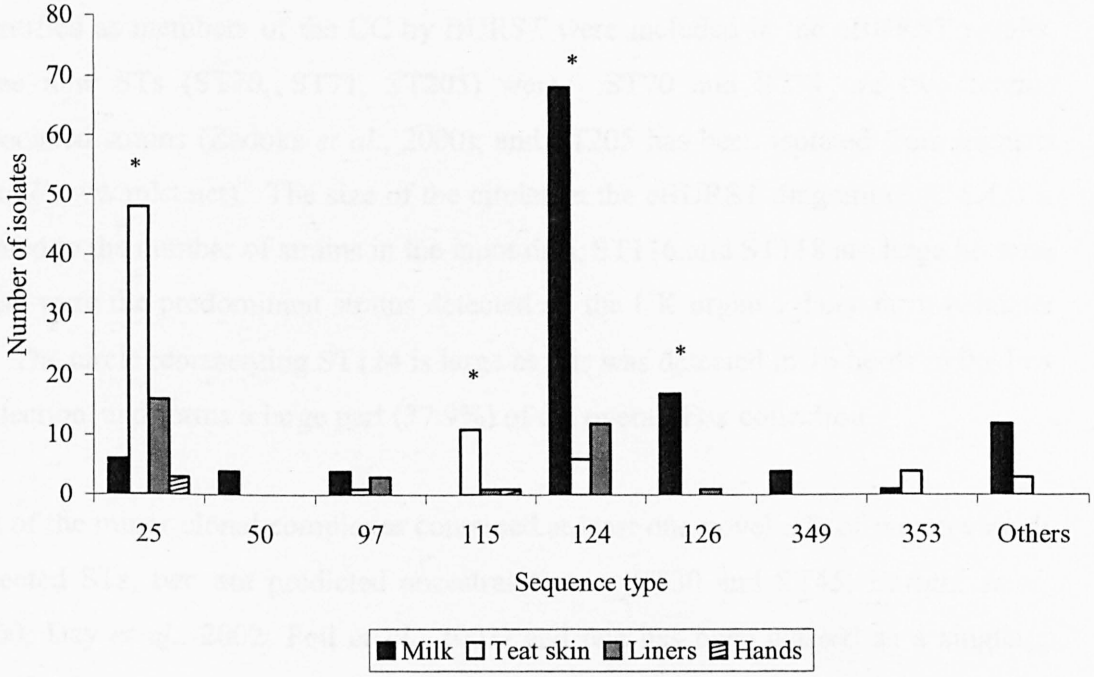


Fig. 5.3. Distribution of ST detected in milk, on teat skin, milking machine unit liners and milkers hands, from the Fox collection.
*statistically significant difference ($p<0.05$) in the frequency of isolation from milk and teat skin.

Table 5.4. Numbers of herds in the Fox collection where the same ST was detected in milk and teat skin, milk and liners, and teat skin and liners.

	S. aureus detected in:		
	Milk and teat skin	Milk and liners	Teat skin and liners
No. of herds	14	10	11
Same ST detected	8	7	9
%	57.1	70.0	81.8

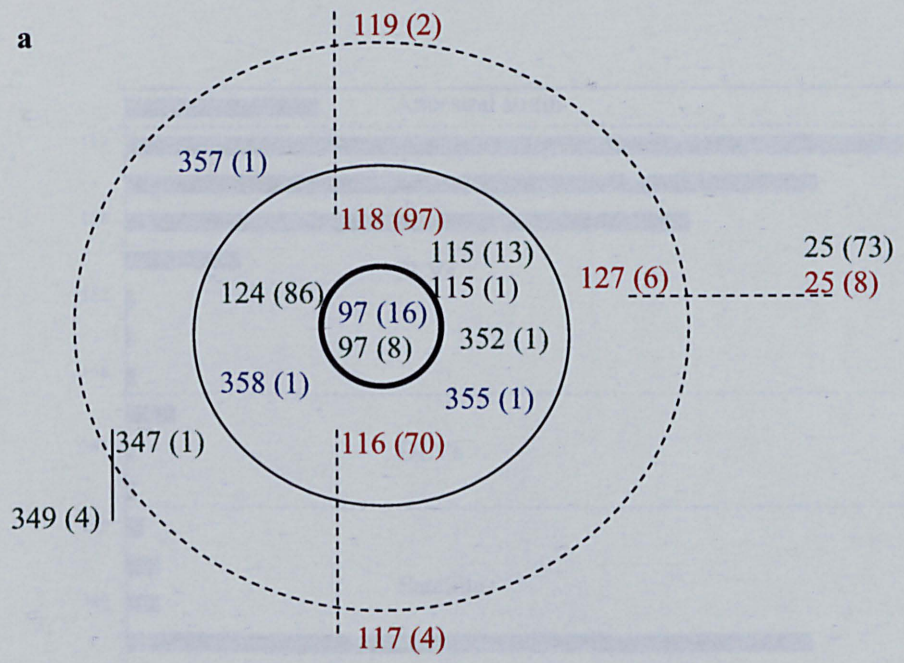
5.3.4 Identification of clonal complexes

The BURST analysis (Fig. 5.4) consisted of 450 isolates, 227 from the USA, 202 from the UK (191 from the organic farm collection (Chapter 4), and 11 from non-organic Somerset dairy farms (Dr. A. J. Bradley)), 20 from Chile and the reference strain *S. aureus* Newbould 305. One major clonal complex, five minor clonal complexes and five singleton STs were identified.

The predicted ancestor of the clonal complex (CC) was ST97, and was given the prefix 'CC' i.e. CC97. Assignment of the ancestral ST by BURST was consistent with the placing of this ST at an internal node by splits decomposition analysis (Fig. 5.5). Further support for this designation was provided by eBURST analysis of this dataset with the entire MLST database (http://www.mlst.net; Fig. 5.4). ST97 was the predicted primary founder with a bootstrap value of 100%, and whilst not all STs identified as members of the CC by BURST were included in the eBURST results, three new STs (ST70, ST71, ST205) were. ST70 and ST71 are two bovine associated strains (Zadoks *et al.*, 2000), and ST205 has been isolated from humans (http://www.mlst.net). The size of the circles in the eBURST diagram (Fig. 5.4b) is related to the number of strains in the input data, ST116 and ST118 are large because these were the predominant strains detected on the UK organic dairy farm (Chapter 4). The circle representing ST124 is large as this was detected in 16 herds in the Fox collection, and forms a large part (37.9%) of the overall Fox collection.

All of the minor clonal complexes contained at least one novel ST, of the previously detected STs, two are predicted ancestral clones (ST30 and ST45; Enright *et al.*, 2000; Day *et al.*, 2002; Feil *et al.*, 2003) and one has been classed as a singleton (ST50; Day *et al.*, 2002; Feil *et al.*, 2003). Of the five singletons, three were represented by more than one isolate (ST126, ST353 and ST9) and were assigned as singleton clones (having no clonal variants). The largest singleton clone was ST126 (16 isolates), ST353 was represented by five isolates and ST9, two isolates. ST9 has been identified as the ancestor of a clonal complex (Day *et al.*, 2002; Enright *et al.*, 2002; Feil *et al.*, 2003), whilst ST126 and ST353 are previously undescribed.

The STs most frequently recovered from milk (ST124) and teat skin (ST25) in the Fox collection, were members of CC97. Of the eight most common STs detected, five (ST25, ST97, ST115, ST124 and ST349) were in the CC, one was part of a minor clonal complex (ST50), and two were singleton clones (ST126 and ST353).



Minor clonal complexes

30 (6) —— 285 (1)

122 (1) —— 45 (2)

348 (1) —— 50 (4)

350 (3) —— 354 (2)

351 (1) ------ 151 (9)

Singletons

126 (18)

353 (5)

9 (2)

356 (1)

1 (1)

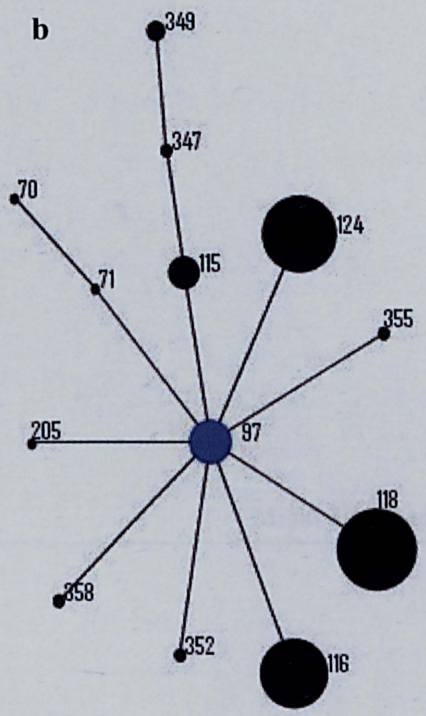


Fig. 5.4. Analysis of bovine *S. aureus* by (a) BURST, (b) eBURST, and (c) [next page] distribution of isolates within the clonal complex, minor clonal complexes and singletons.
BURST analysis: Green numbers denote isolates from the USA, red numbers the UK, blue numbers Chile and the black number is the reference strain *S. aureus* Newbould 305 (NCIMB 702892). The numbers in parentheses are the numbers of isolates of that ST detected. The predicted clonal ancestor is shown in the central ring, single locus variants (SLVs) are shown in the middle (solid) ring, and double locus variants (DLVs) are shown in the outer (dashed) ring. More distantly related STs are classed as satellites, and their relationships are shown as a solid line (single locus difference) or a dashed line (double locus difference).
eBURST analysis: Numbers displayed are sequence types. The blue circle is the primary founder, the size of all circles is relative to the number of each ST in the input data. All lines represent a single locus difference

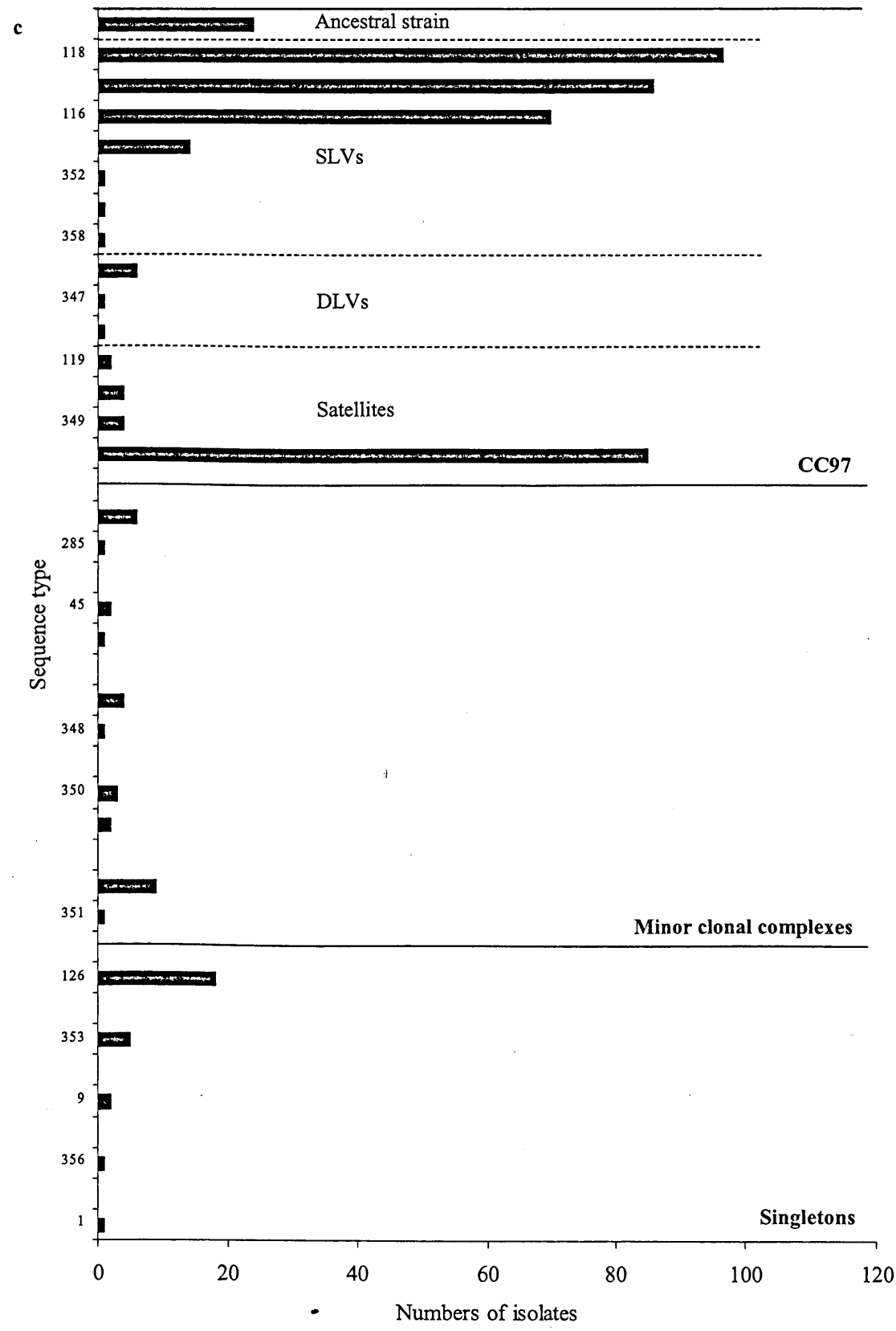


Fig. 5.4c. Distribution of isolates within the clonal complex, minor clonal complex and singletons.

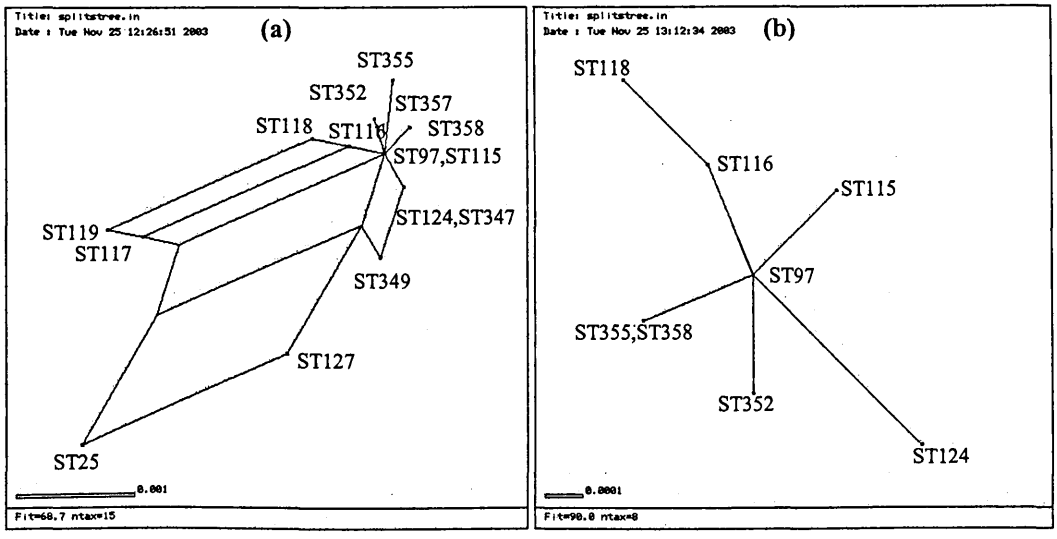


Fig. 5.5. Splits graphs of (a) CC97 and (b) ST97 and it's SLVs.

5.3.5 Estimates of recombination within CC97

As it was possible to assign strains to a clonal complex and determine the most likely ancestor, it was possible to estimate whether SLVs arose by recombination or mutation. CC97 provided seven SLVs for analysis (Table 5.5).

Table 5.5. Allelic variants of the SLVs of CC97.

ST of ancestor	SLV ST	Variable locus	Ancestral allele	SLV Allele	No. nuc. differences[a]	Other clones with SLV allele	Amino acid change
97	115	*yqiL*	3	53	1	ST347, ST349*	Non-synon.
97	116	*tpi*	5	59	1	None	Synonymous
97	118	*tpi*	5	60	2	None	Synonymous
97	124	*gmk*	1	37	2	ST347, ST349*	Non-synon.
97	352	*aroE*	1	78	1	None	Non-synon.
97	355	*arcC*	3	49	2	None	Non-synon.
97	358	*aroE*	1	81	1	None	Synonymous

[a]Number of nucleotide differences.
* SLV allele can be found in other STs within CC97, but not outside CC97.
ST=sequence type, SLV=single locus variant, Non-synon.=non-synonymous amino acid change.

Four of the SLVs had alleles which differed at one nucleotide site from the ancestral ST, three were unique to their ST which is characteristic of point mutation, and all four were unique to CC97. Conversely the alleles of the ancestor are present in more than one CC, supporting the assignment of ancestral and derived STs. The remaining three SLVs contained alleles which differed at two nucleotide sites, of these, two were unique to their ST and all three were unique to CC97. However *tpi*60 differs from *tpi*59 by only a single nucleotide polymorphism, and it is probable this allele

[*tpi*60] arose as a point mutation of *tpi*59 rather than recombination from *tpi*5, this is supported by the splits graph of ST97and it's SLVs (Fig. 5.5b).

The d_N/d_S ratios were calculated for all seven loci, and were less than one. Table 5.6 displays the ratios for the STs in CC97, all bovine strains detected, and the human isolates included in Fig. 5.6.

Table 5.6. d_N/d_S ratios for bovine and human STs.

Locus	CC97	Bovine strains	Human strains
arcC	0.2112	0.2172	0.1507
aroE	0.1374	0.1909	0.1429
glpF	0.0000	0.0856	0.0608
gmk	n/a	0.1680	0.1020
pta	0.6162	0.2081	0.1218
tpi	0.0000	0.1523	0.1855
yqiL	0.1197	0.1454	0.1257
Average	0.1808	0.1668	0.1271

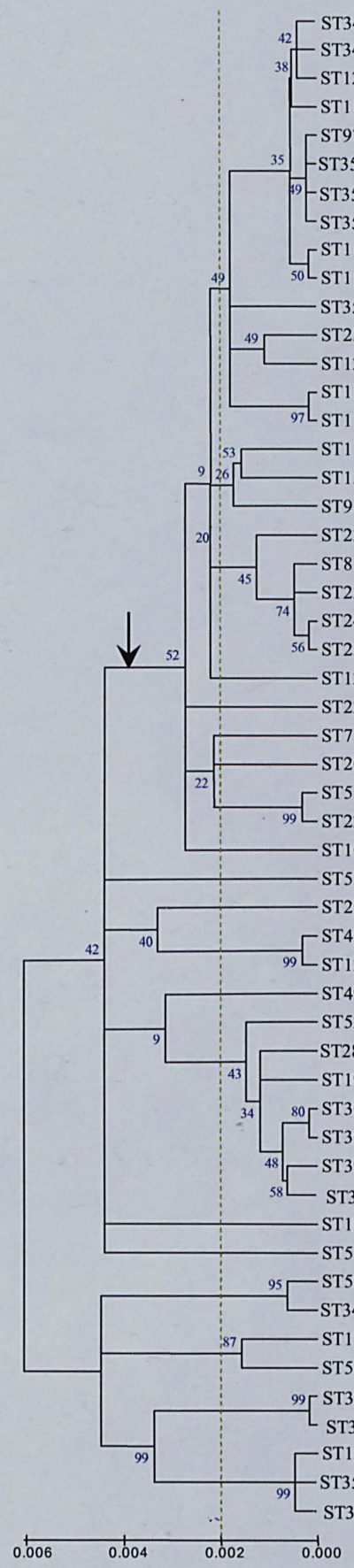
CC97=clonal complex 97

5.3.6 Phylogenetic analysis of *S. aureus*

All the STs in CC97 (Fig. 5.4) are grouped together on a dendrogram of bovine and selected human associated STs (Fig. 5.6). The human strains included have been identified as the ancestral STs of the 11 major MRSA clones (Enright *et al.*, 2002), form the ancestral clones of a human population (Day *et al.*, 2002), or are distantly related STs (singletons) of these analyses (Day *et al.*, 2002; Enright *et al.*, 2002). Clustering of isolates based on host (bovine/human) is demonstrated, and using a cut-off value of 0.002 substitutions/nucleotide site differentiates the members of CC97 from all other STs. This cut-off value also indicates the possible presence of further bovine associated CCs associated with wide geographic spread (ST151/ST351/ST356), or restricted isolates (ST350/ST354). Further analysis of bovine strains will provide greater insight into these and possibly other putative CCs.

The relationships between the alleles detected at each locus and the country and site of isolation are displayed in the locus trees in Appendix 3. Most of the trees support the branching structure of the dendrogram of STs (Fig 5.6). The 'conserved node' (indicated by the arrow) detected by Feil *et al.* (2003) can be placed on Fig. 5.6 by comparing the ST investigated and those on the current tree, and splits the STs

Notes to accompany Fig. 5.6.
The dendrogram was constructed using the Neighbour-joining method in *MEGA* version 2.1 (Kumar *et al.*, 2001), and was based on the seven concatenated allele sequences, in the order *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, *yqiL*. The arrow represents the conserved node as identified by Feil *et al.* (2003). the numbers in blue on the dendrogram are bootstrap values. All information in blue in the table relates to *S. aureus* isolated from cows, all information in red relates to *S. aureus* isolated from humans. *This was the ST of the *S. aureus* reference isolate Newbould 305. Bov.=bovine associated ST; Hum.=human isolated ST. Milk=*S. aureus* isolated from milk; TS=*S. aureus* isolated from teat skin; liners=*S. aureus* isolated from milking machine unit liners; hands=*S. aureus* isolated from milkers hands; bedding=*S. aureus* isolated from cow bedding; cubicle=*S. aureus* isolated from a cubicle partition; personnel=*S. aureus* isolated from farm personnel. Anc.=identified as an ancestral clone by BURST analysis; GISA=glycopeptide intermediate *S. aureus*; MRSA=methicillin resistant *S. aureus*; MRSA Anc.=MRSA ancestral clone; Sing.=ST identified as a singleton by BURST analysis. Source: 1: Fox *et al.* (1991); 2: Chapter 4; 3: N. Tadich/J. Kruze (University of Austral, Chile); 4: A. Bradley (University of Bristol); 5: Day *et al.* (2002); 6: Enright *et al.* (2002). Clonal complex numbers have been assigned on the basis of BURST analysis results either in the present study or the results of the authors from where the ST were derived; S=singleton; minor=member of a minor group.



Host		n (%)	USA					UK				Chile	Type	Source	Clonal complex
			Milk	TS	Liners	Hands	Bedding	Milk	Liners	Cubicle	Personnel	Milk			
ST347	Bov.	1 (0.2)		1 (1.4)										1	97
ST349	Bov.	4 (0.9)	4 (3.4)											1	97
ST124	Bov.	86 (18.9)	68 (58.6)	6 (8.2)	12 (36.4)									1	97
ST115	*Bov./Hum.	14 (3.1)		11 (15.1)	1 (3.0)	1 (25.0)								1	97
ST97	Bov./Hum.	24 (5.3)	4 (3.4)	1 (1.4)	3 (9.1)							16 (80.0)	Sing.	1,3,5	97
ST352	Bov.	1 (0.2)	1 (0.9)											1	97
ST355	Bov.	1 (0.2)										1 (5.0)		3	97
ST358	Bov.	1 (0.2)										1 (5.0)		3	97
ST118	Bov.	97 (21.3)						94 (47.7)	2 (40.0)	1 (100.0)				2	97
ST116	Bov.	71 (15.6)						70 (35.5)	1 (20.0)					2	97
ST357	Bov.	1 (0.2)										1 (5.0)		3	97
ST25	Bov./Hum.	85 (18.7)	6 (5.2)	48 (65.8)	16 (48.5)	3 (75.0)		10 (5.1)	2 (40.0)				Anc.	1,2,5	97
ST127	Bov.	6 (1.3)						6 (3.0)						2	97
ST117	Bov.	4 (0.9)						4 (2.0)						2	97
ST119	Bov.	2 (0.4)						2 (1.0)						2	97
ST1	Bov./Hum.	1 (0.2)	1 (0.9)										Anc.	1,5	1
ST15	Hum.												Anc.	5	15
ST9	Bov./Hum.	2 (0.4)						2 (1.0)					Anc.	4,5	9
ST235	Hum.												GISA	6	5
ST8	Hum.												MRSA Anc.	6	8
ST239	Hum.												MRSA Anc.	6	8
ST247	Hum.												MRSA	6	8
ST250	Hum.												MRSA Anc.	6	8
ST126	Bov.	18 (4.0)	17 (14.7)		1 (3.0)									1	
ST22	Hum.												MRSA Anc.	6	22
ST7	Hum.												Sing.	5	S
ST20	Hum.												Sing.	5	S
ST5	Hum.												MRSA Anc.	6	5
ST228	Hum.												MRSA	6	5
ST101	Hum.												Sing.	5	S
ST55	Hum.												Sing.	5	S
ST207	Hum.												Sing.	5	S
ST45	Bov./Hum.	2 (0.4)	1 (0.9)	1 (1.4)									MRSA Anc.	1,6	45
ST122	Bov.	1 (0.2)	1 (0.9)											1	
ST49	Hum.												Sing.	5	S
ST52	Hum.												Sing.	5	S
ST285	Bov.	1 (0.2)	1 (0.9)											1	
ST19	Hum.												Sing.	5	5
ST30	Bov./Hum.	6 (1.3)	1 (0.9)					1 (100.0)		4 (100.0)			Anc./MRSA Anc.	1,2,5,6	30
ST36	Hum.												MRSA	6	30
ST39	Hum.												Anc.	5	30
ST353	Bov.	5 (1.1)	1 (0.9)	4 (5.5)										1	
ST182	Hum.												Sing.	5	S
ST51	Hum.												Anc.	5	51
ST50	Bov./Hum.	4 (0.9)	4 (3.4)										Sing.	1,5	S
ST348	Bov.	1 (0.2)		1 (1.4)										1	
ST17	Hum.												Sing.	5	S
ST59	Hum.												Sing.	5,6	S/Minor
ST350	Bov.	3 (0.7)	3 (2.6)											1	
ST354	Bov.	2 (0.4)	2 (1.7)											1	
ST151	Bov.	9 (2.0)						9 (4.6)						4	
ST351	Bov.	1 (0.2)	1 (0.9)											4	
ST356	Bov.	1 (0.2)										1 (5.0)		3	

Fig. 5.6. Number (%) of bovine sequence types detected and their country and site of isolation, in addition to a number of *S. aureus* sequence types isolated from humans.

detected into two groups. However it is more difficult to place on some of the individual locus trees i.e. *arcC* and *tpi* as was reported by Feil *et al.* (2003) for *arcC*.

5.4 Discussion

One of the main aims of this study was to determine the effectiveness of MLST as a technique for typing *S. aureus* of bovine origin from a number of distinct geographical sources, and compare it to PFGE typing. In addition, *S. aureus* isolated from milk, teat skin, milking machine unit liners, milkers hands, dairy cow bedding and humans were compared. A further aim was to perform a preliminary analysis of the population and evolutionary biology of *S. aureus* of bovine origin.

The discriminatory power of MLST is similar to other methods of typing *S. aureus* of bovine origin (Aarestrup *et al.*, 1995) and the typeability and Simpson's index of discrimination (D) for the Fox collection was comparable to those obtained with PFGE, phage, and binary typing, although inclusion of PFGE subtypes resulted in a significantly higher index of discrimination (Zadoks *et al.*, 2002). Comparison of the discriminatory power between MLST (D=0.74, 95% CI=0.61-0.87) and binary typing (D=0.86, 95% CI=0.81-0.90) for the Fox collection must be interpreted with caution however as only 142 isolates from the collection were binary typed (Zadoks *et al.*, 2002).

Comparison of MLST with PFGE demonstrated high overall agreement, although PFGE was able to differentiate isolates of ST124, but this could not be correlated to herd or site of isolation. The high level of concordance between MLST and PFGE has been demonstrated for *S. aureus* isolated from humans (Enright *et al.*, 2000; Grundmann *et al.*, 2002*b*; Peacock *et al.*, 2002). It may be that the PFGE types detected in ST124 are evidence for the initial stages in the diversification of this strain, or they may be related to the severity of disease, although no clinical data is available for the Fox collection so this theory cannot be tested. Zadoks *et al.* (2000) however could not correlate PFGE types to the clinical course of infection when examining bovine *S. aureus*. In contrast to the results of binary typing (BT), MLST

and PFGE (Zadoks *et al.*, 2002) results demonstrated congruence with site of isolation data. This suggests MLST and PFGE are more suitable than BT for epidemiological investigations of *S. aureus* of bovine origin.

A combination of typing methods can improve discrimination (Aarestrup *et al.*, 1995), although Grundmann *et al.* (2002*b*) reported that DNA-based typing schemes come to very similar conclusions, and combining them will not increase discrimination to a large extent. Analysis of additional and/or host-specific factors such as the coagulase gene (Schwarzkopf and Karch, 1994; Lange *et al.*, 1999; Raimundo *et al.*, 1999; Su *et al.*, 1999, 2000; Stephan *et al.*, 2001; Schlegelová *et al.*, 2003), toxin production (Fitzgerald *et al.*, 2000; Tollersrud *et al.*, 2000; Akineden *et al.*, 2001; Stephan *et al.*, 2001) and plasmid profiles (Matthews *et al.*, 1992*b*; Aarestrup *et al.*, 1995; Lange *et al.*, 1999; Piccinini and Zecconi, 2001) may however improve the discriminatory power of MLST for large-scale molecular epidemiological analysis of *S. aureus* of bovine origin. Ideally any additional investigations would produce unambiguous results and maintain the transferability of MLST, as is required by a library typing technique (Struelens *et al.*, 1998).

The adoption of a single typing technique for *S. aureus* would allow comparison of strains from different collections, and putatively identify nationally and internationally important clones which could be used in infection studies, possibly leading to improved control. Such a harmonised protocol for typing MRSA has been developed using PFGE (Murchan *et al.*, 2003) and binary typing (van Leeuwen *et al.*, 2002). However, these are not without drawbacks as the interpretation of gel banding patterns is required for PFGE, and only a limited number of laboratories are able to carry out the typing (van Leeuwen *et al.*, 2002; Murchan *et al.*, 2003). Library typing systems on the other hand, are based on unambiguous data, such as MLST, and permit reproduction of the technique in all laboratories with suitable equipment. This also allows direct comparison of isolates from widely varying sources and geographic locations. The reproducibility of MLST between laboratories cannot be assumed however, and requires further study (Peacock *et al.*, 2002). Errors can arise from sequencing, with a single misclassified nucleotide resulting in the assignment of a new allele and ST. The centralisation of sequence and allelic profile data could prove a major strength of MLST, with the inevitable

rapid advances and improvements in sequencing technology and it's automation, the use of MLST is likely to become more popular (Peacock *et al.*, 2002).

Detection of few strains per farm is indicative of a clonal population and cow to cow transmission (Lam *et al.*, 1996; Lipman *et al.*, 1996; Larsen *et al.*, 2000*a*; Zschöck *et al.*, 2000), though some studies suggested a more environmental style of infection (Joo *et al.*, 2001; Sommerhäuser *et al.*, 2003). These differences may be due to the typing scheme used, but there may also be herd specific variations. In herds with a good contagious mastitis control program, bacteria may be prevented from spreading from cow to cow, and essentially become environmental and unlikely to be eradicated (Larsen *et al.*, 2000*a*; Zecconi *et al.*, 2003).

In the present study the majority (87.4%) of bovine-associated isolates belonged to one clonal complex (CC97) which included isolates from all countries and sites. Two other closely related bovine associated putative CCs were identified using 0.002 substitutions/nucleotide site as a method of defining them, although this arbitrary division was by no means foolproof. This was demonstrated by some STs previously identified as members of different CCs falling into the same group e.g. ST1, ST15 and ST9 are grouped together yet have all been identified as potential ancestral strains by BURST analysis (Day *et al.*, 2002). All the isolates in the potential new CCs came from milk, one (ST151/ST351/ST356) contained isolates from all three countries and the other (ST350/ST354) contained only isolates from the USA. The remaining strains belonged to other non-host-specific CCs or were singletons.

Detection of the majority of isolates within one CC agrees with previous reports (Matthews *et al.*, 1994; Kapur *et al.*, 1995; Fitzgerald *et al.*, 1997; Zadoks *et al.*, 2000) that relatively few widely distributed clones of *S. aureus* which give rise to geographically localised variants are responsible for the majority of cases of bovine IMI. The dominant ST detected in each location varied. This is probably due to the evolution of *S. aureus* in diverse hosts and geographical environments (Su *et al.*, 1999). These findings may have important implications for the control of *S. aureus* mastitis; the most effective strategy may need to be directed against the clones causing disease in each area (Matthews *et al.*, 1994; Fitzgerald *et al.*, 1997; Su *et al.*, 1999). It may be that there are further bovine (and potentially other host species)

associated CCs that have yet to be detected. Further analysis of *S. aureus* isolates from bovine mastitis, and those from other hosts from a broader geographical area will help to bridge this gap in knowledge.

Of the 15 members of CC97, two have previously been described (ST97 and ST25; Enright *et al.*, 2000; Day *et al.*, 2002; Feil *et al.*, 2003). Whilst ST97 [the predicted ancestral ST] was not detected in the UK isolates in this study, it has been detected (and classed as a singleton) in human UK isolates (Day *et al.*, 2002; Feil *et al.*, 2003), and detected in ovine isolates (Carter *et al.*, 2003). ST25 has been identified as the ancestral strain of another CC (CC25).

ST25 is a DLV of a DLV of ST97 and so is only distantly related to the ancestral strain. However, this may be evidence for a pathway of strain evolution, with ST127 forming an intermediary between ancestral strains. This is supported by the unrooted Bayesian tree generated by Feil *et al.* (2003), which displays ST97 on the same branch as ST25. The data suggest four locus changes occurred by recombination, three resulting in non-synonymous amino acid changes. Non-synonymous evolution of bovine *S. aureus* is supported by Herron *et al.* (2002) who detected elevated rates of non-synonymous amino acid changes in bovine, compared to human strains. The variation in clones able to infect cows and humans is similar to the clonal diversity demonstrated by *Streptococcus pneumoniae* isolates which are able to infect horses or humans (Whatmore *et al.*, 1999).

Analysis of CC diversification generated some unexpected findings, suggesting there is a roughly equal chance of alleles changing by point mutation and recombination. This contrasts with the results of Feil *et al.* (2003) who estimated that alleles are at least 15-fold more likely to change by point mutation than recombination in the early stages of clonal diversification. Evidence of recombination is also provided by the interconnected 'network' of STs in the splits-graph of the CC (Spratt and Maiden, 1999) and because the conserved node identified by Feil *et al.* (2003) could not be placed on all individual locus trees, indicating recombination has occurred in the evolutionary history of *S. aureus*. Non-congruence between trees constructed for house-keeping genes can infer a history of recombination (Spratt and Maiden, 1999), though it may also be evidence for a paucity of phylogenetically informative sites

(Feil *et al.*, 2003). Analysis of coagulase gene variants also supports the theory of recombinational changes occurring in the evolutionary history of *S. aureus* (Carter *et al.*, 2003).

The presence of alleles which have altered by recombination in more than one strain suggests that these alleles have stabilised and are being maintained in the population. It may be that these changes improve the ability of the resulting strains to colonise and infect cows (Herron *et al.*, 2002).

However, these results should be interpreted with caution, as only one CC and seven SLVs were available for analysis, opposed to the eight CCs and 35 SLVs analysed by Feil *et al.* (2003). Identification of the conserved node at each locus was based on seven of the 25 STs investigated by Feil *et al.* (2003) resulting in examination of three to seven alleles per locus, (putative conserved nodes were not displayed on trees where less than five alleles were examined). Although on the full ST dendrogram, 20 of the 25 ST investigated by Feil *et al.* (2003) were present and there is more confidence in assigning the conserved node. The inability to place the conserved node on all individual locus trees may be due to a 'hitch-hiking' effect of putative virulence genes in the vicinity of the MLST gene (Feil *et al.*, 2003). Interestingly CC97 falls within the conserved area of the dendrogram, with the other putative bovine associated CCs outside this area, suggesting ST97 and its descendants have stabilised and adapted to their niche over time.

These findings may indicate a slightly altered population structure of bovine *S. aureus*. Whether variation is driven by recombination or point mutation, the proteins coded for by the alleles are well conserved as is indicated by the d_N/d_S ratios (Dingle *et al.*, 2001; Herron *et al.*, 2002) for the individual genes. This would be expected though as genes selected for MLST are subject to slow genetic change over time (Maiden *et al.*, 1998). Further work with bovine isolates will provide more information on this aspect of study.

The relationship between site of isolation and ST for the Fox collection is statistically significant. Based on phage typing, Fox *et al.* (1991) suggested teat skin and milking machine unit liners were an important fomite for *S. aureus* IMI, but MLST results do

not support this hypothesis. There was some cross-over of strains between sites, as reported by Zadoks *et al.* (2002) using PFGE and BT. This may indicate that teat skin is a minor source of strains causing IMI, that there was some cross-contamination of the original milk samples, or that MLST was not sufficiently discriminatory. This indicates that liners can act as fomites for both milk and teat skin associated *S. aureus*, although transmission from teat skin to the mammary gland seems rare. This supports the theory that some strains of *S. aureus* demonstrate not just host-, but organ-specificity (Zadoks *et al.*, 2002, Booth *et al.*, 2001).

S. aureus Newbould 305 was first detected in 1958 (Prasad and Newbould, 1968) as a cause of clinical mastitis, and has been used ever since in challenge trials (e.g. Brooks and Barnum, 1984*b*; Schukken *et al.*, 1999) to simulate infections with a naturally occurring species. Infections with *S. aureus* Newbould 305 are known to be relatively gentle infections with a moderate increase in somatic cells in milk, virtually no clinical signs and very high cure rates after therapy (Schukken *et al.*, 1999). The Newbould 305 strain was determined to be ST115, a strain detected on only four farms and associated with teat skin (in this collection). Out of a total of 116 *S. aureus* milk isolates from 28 herds, not one was ST115. This may be indicative of the atypical nature of *S. aureus* Newbould 305 as a representation of udder pathogenic *S. aureus* strains. Typically intra-mammary *S. aureus* infections are characterised by high but variable SCC (Shoshani *et al.*, 2000; de Haas *et al.*, 2002), persistence (Larsen *et al.*, 2000*a*, Chapter 3,4), variable shedding of bacteria (Sears *et al.*, 1990; Buelow *et al.*, 1996*a*) and low cure rates (Craven and Anderson, 1984; Barrio *et al.*, 2000; Hébert *et al.*, 2000). Whilst *S. aureus* Newbould 305 is a convenient strain for challenge trials, it may not be representative of more typical *S. aureus* IMI. It may be wise to catalogue the strains of importance today for use in challenge studies, rather than relying on a strain that is now classified as a skin isolate. Potential strains from the Fox collection, which could be used for this purpose are ST97, ST124 and ST126. As it is now 16 years since this collection was made, it may be wiser to analyse current isolates to determine strains of interest. This could be done by investigating strains held in national and international collections as this would allow analysis of more widespread isolates. The latter two strains (ST124 and ST126) were the most common detected in milk, but ST97 is the

predicted ancestral strain of these, plus other bovine-associated strains of *S. aureus* and may represent a more relevant target for the development of methods to control *S. aureus* IMI. It would also be interesting to type the isolates of Newbould 305 from different research groups and culture collections worldwide, to investigate whether any variation exists at this level. However, use of a *S. aureus* strain in challenge trials which would cause more typical disease would lead to increased welfare problems for the infected cattle. This aspect therefore requires careful consideration before other strains are used in challenge studies.

The ability of the environment to act as a reservoir of *S. aureus* causing IMI was suggested in one herd, where the same ST (ST30) was detected in bedding and as a cause of IMI. This agrees with Matos *et al.* (1991) and Roberson *et al.* (1998) who demonstrated the possibility of the environment as a reservoir of infection. The relative importance of this however is probably low compared with infected cows.

There were a limited number of human skin isolates in the Fox collection. All human skin isolates were collected from the hands of milkers prior to milking, and therefore were not likely to be the result of contamination during milking. The STs detected were also detected in milk and on teat skin suggesting that milkers hands formed a fomite for the transmission of *S. aureus* to teat skin and the mammary gland. Previous studies investigating the relationships between *S. aureus* isolated from humans and cows have been consistent in agreeing in general with the theory of host-specialisation (Lopes *et al.*, 1990; Zadoks *et al.*, 2000; Carter *et al.*, 2003; Schlegelová *et al.*, 2003). However, most authors also demonstrated that some strains of *S. aureus* were able to infect and/or colonise both humans and cows. In some instances farm workers have been shown to be colonised with strains more akin to those found in the bovine rather than human population (Fox *et al.*, 1991; Larsen *et al.*, 2000*a*; Schlegelová *et al.*, 2003).

5.5 Conclusions and further work

The work presented demonstrates that MLST is suitable for typing *S. aureus* of bovine origin, and that the results are able to differentiate strains isolated from different sites. The results agree with the theory of host specialisation, and a previously undetected clonal complex (CC97) associated with bovine infections has been described. The determination of this previously unidentified CC supports the hypothesis of host-specificity within *S. aureus*. It may be that other, less frequently detected STs from both the bovine and human populations e.g. ST50 which as yet cannot be assigned to a particular CC are more suited to uninvestigated hosts such as sheep, pigs, horses or poultry, which may have associated CCs. Further work is required to determine whether this is the case.

Among the bovine associated strains there is evidence for some organ-specificity. In addition, the widely used *S. aureus* reference strain (Newbould 305) was determined to be a skin isolate. Further work needs to be carried out to determine if this is the case and possibly detect more representative strains for challenge and infection studies.

There is a need for the adoption of a single typing technique for *S. aureus* across countries and continents to allow wider comparison of locally important isolates. This is particularly pertinent (and possible) in the case of bovine associated *S. aureus* as it seems a single clone has achieved widespread distribution within cows giving rise to specific localised variants and predominant strains.

There is a potentially higher level of recombination occurring in bovine, compared with human strains, though this view is based on few data and further analyses of a greater number of bovine associated *S. aureus* isolates is required.

A putative intermediary pathway of *S. aureus* evolution from a human to bovine infecting clone has been suggested (ST127), although more work is required to determine the feasibility of this hypothesis.

Chapter 6: General discussion and future prospects

The work presented in this thesis aimed to develop and assess the usefulness of a method which permitted the rapid detection of *S. aureus* in milk, and assess the feasibility of MLST as a method for strain typing bovine associated *S. aureus*.

Whilst the rapid and specific detection of bacteria in milk for diagnosis of IMI is desirable, the method developed (Chapter 2) would probably be of more use in the food industry as a method of determining the contamination rate of bulk milk, or experimentally to follow the progression of an IMI. PCR-based techniques are likely to supersede techniques involving flow cytometry (FCM) as they require much less initial capital, and are probably more accessible to smaller laboratories.

Use of a multiplex PCR (Phuektes *et al.*, 2001) would allow diagnosis of an IMI caused by staphylococci or streptococci, from a single reaction. This is less likely to generate false-positive results than uniplex PCR as sensitivity is not as low, and it is unlikely the analysis would be performed unless there was some other indication of infection e.g. raised SCC or clinical signs.

These methods however do not isolate bacteria for further analysis, though if the FCM technique was coupled with a cell sorter this may permit rapid diagnosis and isolation of causative pathogens. Although the effects of any labelling/marking procedure on the bacteria would need to be fully elucidated to ensure there were no carry-over effects.

S. aureus is a clonal organism, and this is displayed at the herd level (Lipman *et al.*, 1996; Larsen *et al.*, 2000*a*; Younis *et al.*, 2000; Zschöck *et al.*, 2000), although data also exists to contradict this (Joo *et al.*, 2001; Sommerhäuser *et al.*, 2003). This contradictory evidence may be because of colony selection, each time picking a different strain for analysis, or effective control preventing cow to cow spread of infection. Whilst the former is unlikely, preventing the spread of infection has been demonstrated (Chapter 3). On the farms with sporadic infection patterns, even though cow to cow spread has been controlled, infection is still occurring, suggesting

that there may be another reservoir of infection. Environmental strains of *S. aureus* have been demonstrated, but not thought to form a significant reservoir of infection. Although on farms where contagious spread of *S. aureus* has seemingly been prevented, the environment may form a more significant reservoir than infected cattle.

The advent of strain typing of isolates from IMI has drawn attention to the limitations of pathogen classification schemes, though this has rarely been acknowledged (Zadoks, 2002), and little has been done to try to overcome these effects. It seems reasonable to use strain typing in a more relevant classification scheme. MLST is a suitable library typing technique because it offers unambiguous identification and is completely transferable. Schemes have been developed or are under development for the main mastitis pathogens (Enright *et al.*, 2000; Jones *et al.*, 2003; http://www.mlst.net). Classification allows comparison of strains, from an international database, isolated worldwide. Alongside data concerning source, level of infection and possibly herd and management factors, this would provide the basis of a more comprehensive pathogen classification scheme, generate data to analyse the evolutionary and population biology of mastitis pathogens, and allow comparison of strains associated with human disease. There would also be the potential to monitor the spread of new strains and/or clones.

Further MLST analysis of *S. aureus* isolated from cattle from a number of geographical regions would provide more information on their epidemiology and population biology. This would be best achieved through a large international collaboration. *S. aureus* has been isolated previously in regions as diverse as Argentina, Australia, Brazil, Czech Republic, Denmark, Ethiopia, Finland, Germany, Ireland, Israel, Italy, Japan, Korea, The Netherlands, Norway, Sweden, UK, USA (Lopes *et al.*, 1990; Matsunaga *et al.*, 1993; Aarestrup *et al.*, 1995, 1997; Lipman *et al.*, 1996; Myllys *et al.*, 1997; Annemüller *et al.*, 1999; Lange *et al.*, 1999; Raimundo *et al.*, 1999; Fitzgerald *et al.*, 2000; Larsen *et al.*, 2000*a,b*; Sordelli *et al.*, 2000; Tollersrud *et al.*, 2000; Younis *et al.*, 2000; Zadoks *et al.*, 2000; Zschöck *et al.*, 2000; Buzzola *et al.*, 2001; Joo *et al.*, 2001; Piccinini and Zecconi, 2001; Takeuchi *et al.*, 2001; Green *et al.*, 2002; Middleton *et al.*, 2002; Workineh *et al.*, 2002; Schlegelová

et al., 2003; Sommerhäuser *et al.*, 2003). Analysis of such a large range of isolates would determine whether differences in *S. aureus* populations exist between herds.

As MLST is transferable, analysis could be performed in separate laboratories as has been demonstrated for MLST analysis of *Campylobacter jejuni* (Dingle *et al.*, 2001). This would remove the need for shipping of potentially dangerous organisms. However it would be wise initially to analyse a selection of isolates in each laboratory to ensure reproducibility, as for PFGE and binary typing of MRSA (van Leeuwen *et al.*, 2002; Murchan *et al.*, 2003). Once repeatable, these laboratories would not only be able to analyse strains from cows, but all potential sources because the technique does not differ by host species.

It would also be of interest to analyse isolates from other species such as sheep (Gonzalo *et al.*, 2002), goats (McDougall, 2000) and camels (Guliye *et al.*, 2002), to determine whether CC97 is host-specific, as demonstrated for some porcine isolates of *C. jejuni* (Manning *et al.*, 2003), or associated with the mammary gland of many production species.

Whilst the presence of different strains in whole animals has been demonstrated, few data exist concerning strain variation within mammary quarters. Studies of intra-mammary epidemiology would benefit from analysis of a greater number of isolates from individual quarters. Data has been presented suggesting some combinations are more likely to occur than others (Chapter 4), though this is far from conclusive. It may be that some strains work in concert, with infections initially established by an invasive strain, which makes the gland more susceptible to infection and colonisation by another more persistent strain. There may also be other interactions, but only more detailed analysis of mammary quarter infections will determine this.

The hypothesis that recombination occurs at increased rates in bovine opposed to human strains cannot be rejected. This may be because as yet only one CC has been identified associated with bovine infections and because of the varying geography and management selection pressures applied to the isolates studied. Recombination may improve the fitness of localised strains in the initial stages of clonal diversification; however further strain evolution may be through single mutational

changes causing, in effect strain 'fine-tuning' as they become more established in mammary quarters.

More data on this aspect of *S. aureus* strain evolution could be determined by analysis of historical strains and/or collections. This may also produce evidence for putative evolutionary pathways or intermediaries in the evolution of bovine strains of *S. aureus* from human-associated clones.

Identification of *S. aureus* Newbould 305 as a skin-associated isolate has important implications for future experimental studies of *S. aureus* IMI. Whilst it offers welfare benefits, other strains may be more suitable as discussed (Chapter 5).

In conclusion, the data presented in this thesis demonstrate that a large number of bovine *S. aureus* IMI are associated with strains belonging to one CC; further analyses are required at the animal and international level to fully understand the epidemiology and population biology of *S. aureus*. However, the identification of a clonal ancestor of strains associated with IMI from two continents may help to determine targets for future control of *S. aureus* IMI.

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Appendix 1: *Reprint*: Letter to the Editor

Smith, E.M., Green, L.E., Mason, D. (2003). Savinase is a bactericidal enzyme. *Applied and Environmental Microbiology*, **69** (1), 719-721.

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 2003, p. 719–721
0099-2240/03/$08.00+0 DOI: 10.1128/AEM.69.1.719–721.2003

Vol. 69, No. 1

Letter to the Editor

Savinase Is a Bactericidal Enzyme

Mastitis is probably the most important disease affecting dairy cows worldwide and has been estimated to cost $1.5 to 2.0 billion/year in the United States alone (4). Although detection methodology continues to improve, microbiological culture is still the recommended method (2) for determining the bacterial content of a milk sample, despite this procedure being both time-consuming and expensive. Consequently, more rapid methods of identification and/or enumeration of bacteria in milk are being developed (3, 5). Many of these techniques, e.g., PCR-based methods, can be completed in a matter of hours, but they lack the specificity to be able to determine the bacterial species present or are unable to enumerate the bacteria present. There can also be a problem with techniques that require a period of enrichment prior to analysis.

The instrument of choice for overcoming problems of enumeration is a flow cytometer (FCM). In the paper by Gunasekera and colleagues published in *Applied and Environmental Microbiology* in March 2000, a method for the rapid detection and enumeration of bacteria in milk, without the need for enrichment, was outlined (1). Gunasekera et al. stated that an enzymatic treatment was required to remove and/or modify protein globules present in milk to enable the distinction of bacteria. Two alternative enzymes were suggested for this purpose, proteinase K (Sigma-Aldrich, Dorset, England) and savinase (Novozymes, Bagsvaerd, Denmark), a protease enzyme developed for the detergent industry.

However, when we used savinase to recreate and develop this method to identify *Staphylococcus aureus* in milk, we found that bacterial recovery from the original milk sample was below 50% (data not shown). Our investigations led us to determine the effect (if any) that savinase itself was having on *S. aureus*. The results indicated that two separate batches of savinase were immediately bactericidal towards 12 strains of *S. aureus* in pure culture. These strains included the one used by Gunasekera et al. (NCTC 4163), the Newbould 305 strain (NCIMB 702892), and 10 strains isolated from cattle with clinical or subclinical bovine mastitis.

The effects of savinase, proteinase K, and alcalase (Novozymes), the latter being an enzyme designed to break down proteins in milk to produce infant milk formula, on *S. aureus* Newbould 305 inoculated into pasteurized milk were also investigated. Savinase took between 3 and 5 h (at 37°C) to kill all the bacteria in the inoculated milk sample (Fig. 1) and resulted in a significantly lower ($P < 0.001$) bacterial count than the other two treatments after only 60 min. This is well within the time frame (60 to 90 min) of the protocol described by Gunasekera and colleagues.

These results may be due to a difference in the formulations of savinase supplied in the United Kingdom and Australia, and we would be interested to know whether this is the case. However, we would like to point out that, in the United Kingdom, savinase appears to be unsuitable for use in the detection of *S. aureus* in milk.

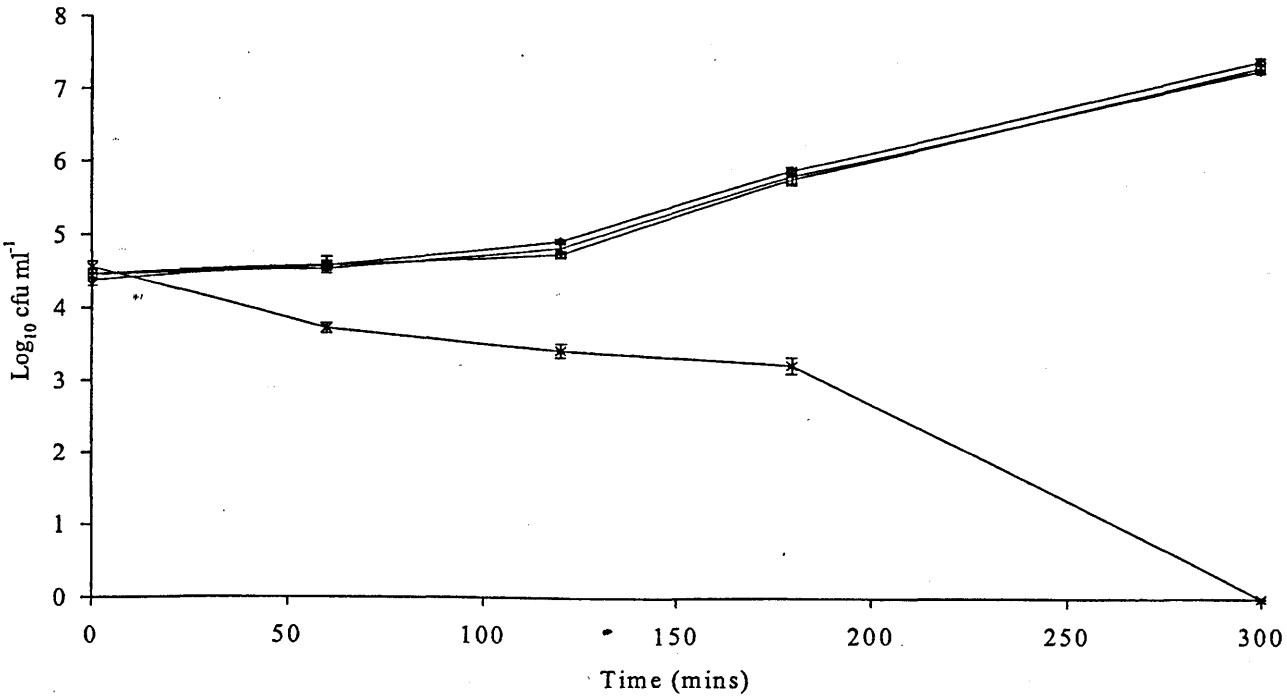

FIG. 1. How protease enzymes can affect the growth of *S. aureus* Newbould 305 in pasteurized milk. Shown are the effects of no enzyme (control) (◇), proteinase K (□), alcalase (+), and savinase (×).

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Author's Reply

This letter is written in response to the Letter to the Editor written by Smith et al., titled "Savinase Is a Bactericidal Enzyme." These authors used our method (1) and standard plate count methods to assess the total count and the viability of savinase-treated *S. aureus* bacteria. Their experiments demonstrated that savinase is a bactericidal enzyme and may not be suitable for clearing milk proteins as a sample preparation procedure for FCM detection and enumeration of *S. aureus* bacteria in milk.

The original article published in *Applied and Environmental Microbiology* (1) described the use of FCM for rapid detection and enumeration of total bacteria in milk. In this method, savinase 16L (type EX; activity, 16 KNPU/g) and proteinase K were used as milk-clearing agents, and the nucleic acid binding fluorescent dye SYTO BC was used for staining bacteria in milk. Ultra-high-temperature-treated (UHT) milk was inoculated with *Escherichia coli* or *S. aureus* cells at concentrations between 10^3 and 10^8 ml^{-1} and analyzed by the FCM and plate count methods. To provide more-realistic data, raw milk samples were analyzed by the FCM method and the results were compared with those obtained by the standard plate count method. Samples used for analysis by the FCM method were subjected to milk clearing, but subsamples used for analysis by the plate count method were not treated with savinase or proteinase K. Results from the FCM method had good correlation with those from the plate count method.

To address the concerns of Smith et al., we have determined, (i) the effects of savinase (10 μl per 100 μl of UHT milk) on total counts and numbers of CFU over time (0 to 300 min) and (ii) the effects of a higher concentration of savinase (50 μl per 100 μl of UHT milk) on both total counts and numbers of CFU determined by FCM after incubation of the sample for 40 min. Using the concentration recommended for UHT milk (10 μl per 100 μl of UHT milk) (1), we are unable to reproduce the effects of savinase described by Smith et al., either on total counts or on numbers of CFU determined by FCM (Fig. 1). Unfortunately, Smith et al. did not provide the FCM results nor did they indicate the concentrations of savinase that they used for comparison. Using the higher concentration recommended for raw milk (50 μl per 100 μl), we found no reduction of the total count obtained by FCM but found a reduction in the number of CFU (Fig. 2). Savinase at even higher concentrations and longer incubation times than those recommended (1) did not decrease the total bacterial count (Fig. 1 and 2).

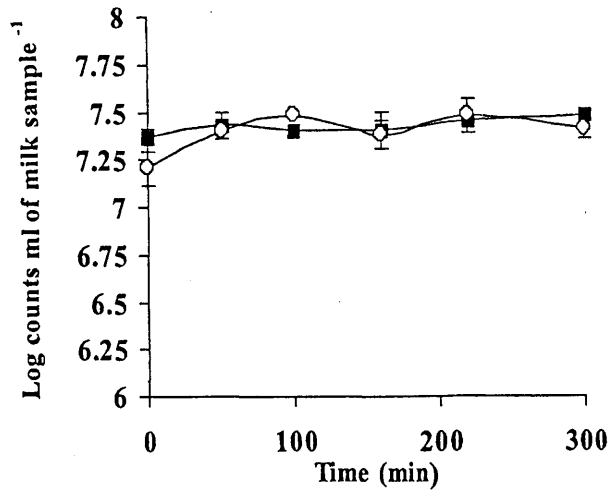

FIG. 1. Effect of savinase on the viability of *S. aureus*. UHT milk was inoculated with *S. aureus*, and 100-μl milk samples were treated with 10 μl of savinase for different time intervals. Samples were processed as described by Gunasekera et al. (1), and the total numbers of bacteria were determined by FCM. Viable counts were determined by the plate count method. Ovals, plate counts; squares, FCM counts.

The effect of the milk-clearing procedure on bacterial viability was not an issue, since the method we described (1) was primarily developed to detect and enumerate the total number of bacteria in milk. SYTO BC stains both live and dead bacterial cells (R. Haugland, *Handbook of Fluorescent Probes and Research Chemicals*. Molecular Probes, Inc. Eugene, Oreg. 1996). Thus, any effect that savinase might have on bacterial colony-forming ability is not going to affect the total count (Fig. 2). Thus, the effect of savinase on colony-forming ability

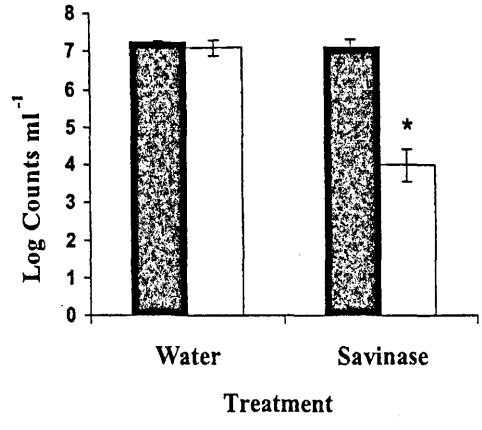

FIG. 2. Effect of 50 μl of savinase on total *S. aureus* populations. UHT milk was inoculated with *S. aureus*, and 100-μl milk samples were treated with 50 μl of savinase for 40 min. Samples were processed as described by Gunasekera et al. (1), and the total numbers of bacteria were determined by FCM. Viable counts were determined by the plate count method. Controls (bacteria in buffer) were treated with sterilized distilled water. Filled bars, FCM counts; open bars, plate counts. Data were analyzed by using Student's *t* test (an asterisk denotes significant difference at a P value of <0.05).

is not an important issue (as described by Smith et al.) for our method (1). The effect of higher concentrations of savinase on CFU is not surprising. In fact, for viability assays, we use alternative milk-clearing agents or low levels of savinase (2).

Dr. Smith and his colleagues have used the numbers of CFU to determine the viability of savinase-treated, stressed bacteria. I would contend that the numbers of CFU do not accurately reflect the viability of all stressed bacteria (3). This is especially the case where cells may be viable but not culturable. Other parameters, such as cell vitality, cell permeability, membrane potential, and enzyme activity, are also useful indicators in measuring cellular activities.

Another point of concern of Dr. Smith and his colleagues is the formulation of savinase supplied in the United Kingdom and Australia. Savinase is available in six different formulations, and for our studies in Australia we used savinase 16L, type EX (declared activity, 16 KNPU/g). Batch-to-batch variation is also worth considering. We would therefore be happy to supply a sample of the savinase we used for the above experiments to Dr. Smith, or we can test the savinase used by Smith et al. We certainly expect that different formulations have different effects on milk proteins and, more importantly, on the bacteria in milk. Therefore, care in choosing the correct enzyme type for milk clearing is important, particularly when viability testing is required.

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Appendix 2: Experimental variations tested to optimise milk clearing method

Table A2.1. Summary and effects of variables tested to optimise milk clearing method.

Expt.	Variable	Variations	Notes	% recovery
1	Buffer concentration	50mM*		90.41
		100mM		90.05
		150mM		89.17
2	Buffer volume	200µl*		90.98
		400µl		87.13
		900µl		86.72
3	Centrifuge	swing-out*		97.66
		fixed-angle		89.24
4	Centrifugation speed	10,000×*g**		96.67
		8,000×*g*		93.47
5	Use of a detergent	none		92.12
		0.1% triton	added before incubation	94.33
		0.1% triton	added after incubation	98.77
6	Buffer	50mM NaCl*		92.77
		0.5% BSA		88.22
7	Centrifugation time	5min		94.97
		10min*		93.01
8	Larger volume	100µl*		91.73
		1000µl	Oak ridge tube	87.01
9	No buffer (larger volume)	no detergent		96.43
		+ 0.1% triton	added before incubation	96.50
		+ 0.1% chaps	added before incubation	93.94
		+ 0.1% NP40*	added before incubation	99.05
10	Freezing	none*		92.60
		-20°C		92.82
		-80°C		92.19
11	NP40 concentration	0.1%*		107.15
		0.2%		107.07
		0.3%		109.34
		1.0%		108.19
12	Enzyme concentration	10%*		106.73
		20%		106.20
		30%		110.61
		40%		108.78
13	Naturally infected milk			114.33
14	Spiked milk			112.90
15	FCM analysis	(no NP40)		95.36

*Chosen variable, mM=millimolar, µl=microlitre, ×*g*=centrifugal force, BSA=bovine serum albumin, FCM=flow cytometry.

Appendix 3: Environmental and body site samples by visit

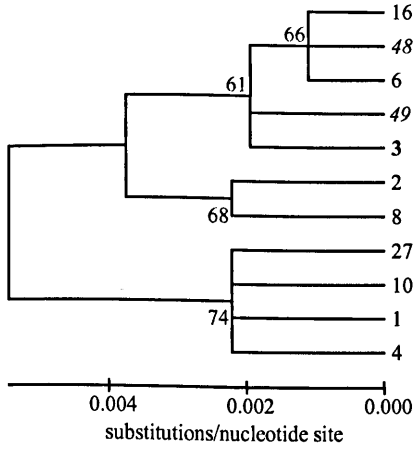
Table A3.1. Numbers of non-milk samples and results of bacteriology by visit.

	Visit number										
Sample/organism	1	2	3	4	5	6	7	8	9	10	Total (%)
Clusters	**1**	**1**	**6**	**7**	**7**	**6**	**6**	**5**	**5**	**6**	**50**
CPS	0	0	0	0	2	0	0	2	0	1	5 (10.0)
CNS	1	0	4	4	7	4	3	2	0	6	31 (62.0)
Streptococci	0	1	0	0	2	2	0	1	1	5	12 (24.0)
Gram-negative	1	1	1	0	2	1	6	4	3	2	21 (42.0)
Water	**0**	**8**	**8**	**8**	**8**	**7**	**7**	**0**	**0**	**0**	**46**
CPS		0	0	0	0	0	0				0 (0.0)
CNS		1	5	4	3	1	0				14 (30.4)
Streptococci		0	3	0	0	0	0				3 (6.5)
Gram-negative		8	0	3	0	4	6				21 (45.7)
Environment	**0**	**18**	**19**	**19**	**17**	**19**	**17**	**16**	**15**	**12**	**152**
CPS		0	1	0	0	0	0	0	0	0	1 (0.7)
Streptococci		0	0	1	1	1	0	0	0	0	3 (2.0)
Animals	**0**	**3**	**1**	**2**	**2**	**2**	**2**	**3**	**2**	**3**	**20**
CPS		0	0	0	0	1	0	0	1	0	2 (10.0)
Personnel	**0**	**6**	**4**	**6**	**6**	**4**	**4**	**4**	**6**	**6**	**46**
CPS		0	1	0	1	0	0	0	1	1	4 (8.7)
Body sites	**0**	**30**	**138**	**0**	**36**	**54**	**60**	**0**	**0**	**0**	**318**
CPS		0	0	0	0	0	0	0	0	0	0 (0.0)
Streptococci		0	0	0	1	0	1	0	0	0	2 (0.6)
Air	**0**	**0**	**5**	**5**	**5**	**5**	**5**	**0**	**0**	**0**	**25**
CPS			0	0	0	0	0				0 (0.0)
CNS			4	0	3	4	5				16 (64.0)
Gram-negative			0	0	0	1	0				1 (4.0)
Feed	**0**	**0**	**8**	**9**	**6**	**6**	**6**	**0**	**0**	**0**	**35**
CPS			0	0	0	0	0				0 (0.0)
Streptococci			0	3	0	0	0				3 (8.6)
Bedding	**0**	**0**	**9**	**9**	**8**	**3**	**1**	**0**	**0**	**0**	**30**
CPS			0	0	0	0	0				0 (0.0)
Streptococci			0	2	0	0	0				2 (6.7)

CPS=coagulase positive staphylococci, CNS=coagulase negative staphylococci.

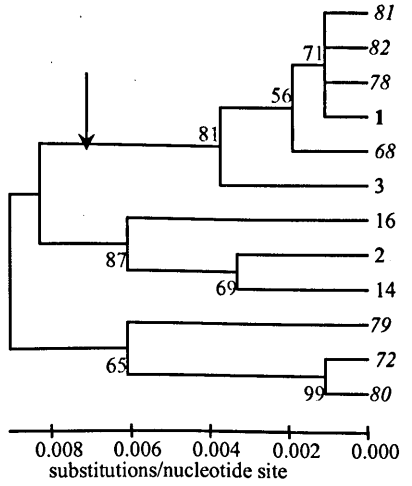
Appendix 4: Individual MLST locus trees for bovine *S. aureus* strains

arcC



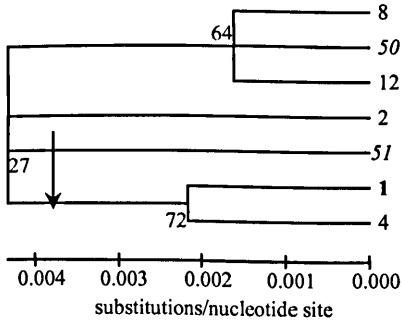
	USA			UK	Chile	Grand
Milk	TS		Liners	Milk	Milk	Total
4 (3.4)	1 (1.4)					5 (1.1)
					1 (5.0)	1 (0.2)
6 (5.2)				9 (4.6)		15 (3.4)
					1 (5.0)	1 (0.2)
90 (77.6)[a]	19 (26.0)[b]		17 (51.5)[b]	172 (87.3)	18 (90.0)	316 (72.0)
2 (1.7)						2 (0.5)
1 (0.9)	4 (5.5)					5 (1.1)
1 (0.9)						1 (0.2)
1 (0.9)	1 (1.4)					2 (0.5)
5 (4.3)						5 (1.1)
6 (5.2)[a]	48 (65.8)[b]		16 (48.5)[c]	16 (8.1)		86 (19.6)
116	73		33	197	20	439
(100.0)	(100.0)		(100.0)	(100.0)	(100.0)	(100.0)

aroE



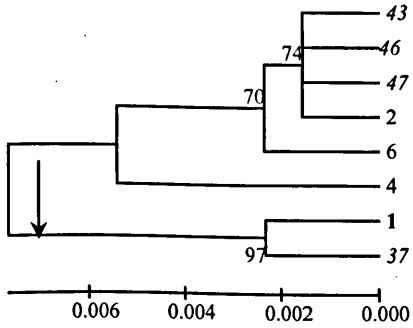
	USA			UK	Chile	Grand
Milk	TS		Liners	Milk	Milk	Total
					1 (5.0)	1 (0.2)
					1 (5.0)	1 (0.2)
1 (0.9)						1 (0.2)
83 (71.6)[a]	67 (91.8)[a]		32 (97.0)[b]	186 (94.4)	17 (85.0)	385 (87.7)
17 (14.7)			1 (3.0)			18 (4.1)
				2 (1.0)		2 (0.5)
4 (3.4)	1 (1.4)					5 (1.1)
3 (2.6)	4 (5.5)					7 (1.6)
2 (1.7)	1 (1.4)					3 (0.7)
5 (4.3)						5 (1.1)
1 (0.9)				9 (4.6)		10 (2.3)
					1 (5.0)	1 (0.2)
116	73		33	197	20	439
(100.0)	(100.0)		(100.0)	(100.0)	(100.0)	(100.0)

glpF



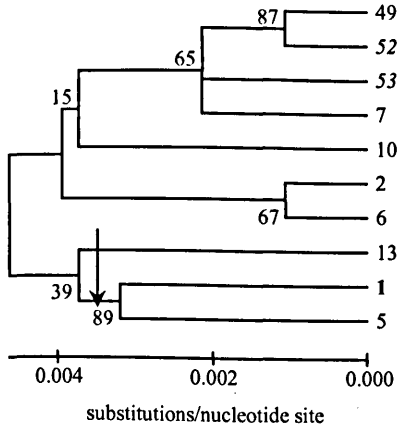
	USA			UK	Chile	Grand
	Milk	TS	Liners	Milk	Milk	Total
8	2 (1.7)	1 (1.4)				3 (0.7)
50	1 (0.9)				1 (5.0)	2 (0.5)
12	4 (3.4)	1 (1.4)		9 (4.6)		14 (3.2)
2	3 (2.6)	4 (5.5)				7 (1.6)
51	5 (4.3)					5 (1.1)
1	95 (81.9)[a]	19 (26.0)[b]	17 (51.5)[b]	172 (87.3)	19 (95.0)	322 (73.3)
4	6 (5.2)[a]	48 (65.8)[b]	16 (48.5)[c]	16 (8.1)		86 (19.6)
	116	73	33	197	20	439
	(100.0)	(100.0)	(100.0)	(100.0)	(100.0)	(100.0)

gmk



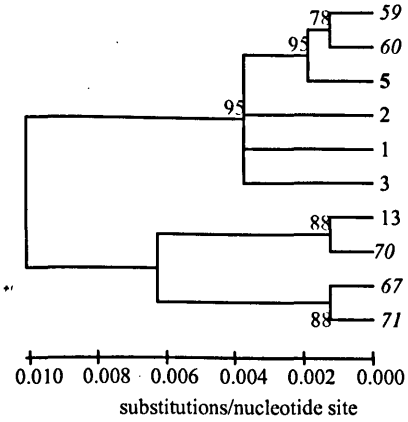
	USA			UK	Chile	Grand
	Milk	TS	Liners	Milk	Milk	Total
43	1 (0.9)			9 (4.6)	1 (5.0)	11 (2.5)
46	1 (0.9)	4 (5.5)				5 (1.1)
47	5 (4.3)					5 (1.1)
2	5 (4.3)					5 (1.1)
6	2 (1.7)	1 (1.4)				3 (0.7)
4	18 (15.5)	1 (1.4)	1 (3.0)			20 (4.6)
1	12 (10.3)[a]	60 (82.2)[b]	20 (60.6)[a]	188 (95.4)	19 (95.0)	299 (68.1)
37	72 (62.1)[a]	7 (9.6)[b]	12 (36.4)[b]			91 (20.7)
	116	73	33	197	20	439
	(100.0)	(100.0)	(100.0)	(100.0)	(100.0)	(100.0)

pta



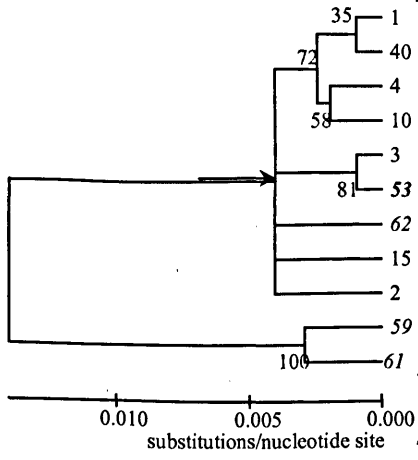
	USA		UK	Chile	Grand
Milk	TS	Liners	Milk	Milk	Total
			9 (4.6)	1 (5.0)	10 (2.3)
1 (0.9)					1 (0.2)
2 (1.7)					2 (0.5)
3 (2.6)					3 (0.7)
2 (1.7)	1 (1.4)				3 (0.7)
1 (0.9)	4 (5.5)				5 (1.1)
2 (1.7)					2 (0.5)
4 (3.4)	1 (1.4)				5 (1.1)
95 (81.9)[a]	19 (26.0)[b]	17 (51.5)[b]	172 (87.3)	19 (95.0)	322 (73.3)
6 (5.2)[a]	48 (65.8)[b]	16 (48.5)[c]	16 (8.1)		86 (19.6)
116	73	33	197	20	439
(100.0)	(100.0)	(100.0)	(100.0)	(100.0)	(100.0)

tpi



	USA		UK	Chile	Grand
Milk	TS	Liners	Milk	Milk	Total
			74 (37.6)		74 (16.9)
			96 (48.7)		96 (21.9)
100 (86.2)[a]	67 (91.8)[b]	33 (100.0)[c]	16 (8.1)	19 (95.0)	235 (53.5)
1 (0.9)	4 (5.5)				5 (1.1)
1 (0.9)			2 (1.0)		3 (0.7)
4 (3.4)	1 (1.4)				5 (1.1)
4 (3.4)	1 (1.4)				5 (1.1)
5 (4.3)					5 (1.1)
1 (0.9)			9 (4.6)		10 (2.3)
				1 (5.0)	1 (0.2)
116	73	33	197	20	439
(100.0)	(100.0)	(100.0)	(100.0)	(100.0)	(100.0)

yqiL



	USA			UK	Chile	Grand
	Milk	TS	Liners	Milk	Milk	Total
1	1 (0.9)					1 (0.2)
40	17 (14.7)		1 (3.0)			18 (4.1)
4	6 (5.2)[a]	48 (65.8)[b]	16 (48.5)[c]	16 (8.1)		86 (19.6)
10				2 (1.0)		2 (0.5)
3	73 (62.9)[a]	7 (9.6)[b]	15 (45.5)[b]	170 (86.3)	18 (90.0)	283 (64.5)
53	4 (3.4)[a]	12 (16.4)[b]	1 (3.0)[a]			17 (3.9)
62					1 (5.0)	1 (0.2)
15	4 (3.4)	1 (1.4)				5 (1.1)
2	5 (4.3)	5 (6.8)				10 (2.3)
59	1 (0.9)			9 (4.6)	1 (5.0)	11 (2.5)
61	5 (4.3)					5 (1.1)
	116	73	33	197	20	439
	(100.0)	(100.0)	(100.0)	(100.0)	(100.0)	(100.0)

Fig. A4.1. Individual gene trees for each of the seven MLST loci and the number (%) of isolates detected in each country and site of isolation.
The trees were constructed using the Neighbour-joining method in *MEGA* version 2.1 (Kumar *et al.*, 2001). The arrows represent the conserved nodes as determined by Feil *et al.* (2003). Values in the same row with different superscripts differ significantly ($p<0.05$). Numerical values displayed on the tree in blue are bootstrap values. Milk=milk isolates; TS = teat skin isolates; liners=milking machine unit liner isolates. The allele identified in bold on each tree is the allele found in the reference strain of *S. aureus* (Newbould 305). The alleles identified in italics on each tree are the novel alleles discovered during the course of this work.