Bovine *Staphylococcus aureus* mastitis: strain recognition and dynamics of infection

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Summary. Restriction enzyme fragmentation pattern (REFP) analysis was used to recognise *Staphylococcus aureus* strain variation in naturally occurring bovine subclinical mastitis. Multiple colony REFP analysis identified eight distinct strains of *S. aureus* in addition to the original strains A and B that were infused via the intramammary route, indicating that individual quarters of the udder may be colonised simultaneously by more than one strain of *S. aureus*. Examination of multiple colonies per milk sample may benefit bacterial strain recognition as an epidemiological tool in mastitis investigations. The dynamics of intramammary infection were determined using a novel double crossover experimental challenge. Quarters remained persistently infected for several weeks following challenge in all four cows, irrespective of the challenge strain. This indicated that no alteration of the original subclinical infection, including the possible induction of clearance of the quarters infected with *S. aureus*, or replacement of the original strain by the infused strain was induced by challenge. The persistent subclinical infection in all four animals supports previous reports on the chronicity of *S. aureus* intramammary infection in dairy cows.

Keywords: Mastitis, dairy cows, *Staphylococcus aureus*, strain typing, restriction enzyme.

Mastitis causes greater financial loss to the dairy industry than any other disease (De Graves & Fetrow, 1993) and is recognised as one of the major problems that adversely affects dairy cow welfare (Menzies *et al.* 1995). Subclinical mastitis, caused by *Staphylococcus aureus*, has been associated with a substantial reduction in farm income (Blosser, 1979; Rebhun, 1995). The infectiousness of staphylococci is dependent on many factors, such as the number of bacteria, the route of infection, or the production of virulence factors (Lee, 1996). Matsunaga *et al.* (1993) compared the distribution of virulence factors among *S. aureus* isolates from peracute, acute and chronic bovine mastitis cases and correlated the results with clinical

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presentation. These authors concluded that all *S. aureus* isolates from peracute cases produced TSST-1, staphylococcal enterotoxin C, α-haemolysin and β-haemolysin, whereas none of the peracute isolates produced clumping factor or protein A, which were more often associated with chronic mastitis. However, there was no obvious variation in production of virulence factors between isolates from acute cases and isolates from chronic cases, which is in agreement with the findings of Jonsson & Holmberg (1981).

S. aureus avoids elimination from the udder by a number of mechanisms, including the ability to survive inside white blood cells and to outlive these cells, thus surviving within the udder (Bayles *et al.* 1998). Antibiotics have been in widespread use as a mastitis therapy for several decades and are generally effective, although less so against *S. aureus* mastitis due to the ability of the organisms to become sequestered within microabscesses (Craven & Anderson, 1984), the development of antibiotic resistance, and the failure of some antibiotics to penetrate into the phagolysosome (Madgwick *et al.* 1989). Many strains of *S. aureus* produce β-lactamase which renders them resistant to β-lactam antimicrobial agents such as the penicillins (Watts & Salmon, 1997), making antibiotics such as amoxycillin/clavulanic acid, cloxacillin or nafcillin the drugs of choice for intramammary infections caused by *S. aureus* (McKellar, 1991). Improved understanding of the pathogenesis of staphylococcal mastitis will facilitate therapeutic and prophylactic approaches to controlling this disease.

Many genotypic methods have been used to study *S. aureus* strain diversity in cases of bovine mastitis (Robertson *et al.* 1994; Aarestrup *et al.* 1995; Lam *et al.* 1996). Restriction enzyme fragmentation pattern (REFP) analysis after digestion with frequent cutting enzymes has proved valuable in the analysis of both salmonellae (Platt *et al.* 1996) and human *S. aureus* using the restriction enzymes *Hha* I and *Sau* 3A (Brown, 1998). This demonstrated diversity among unrelated strains, homogeneity of REFP among isolates from outbreaks, and also allowed the recognition of molecular variation among strains from extended outbreaks of disease.

Here we report the use of REFP analysis after *Hha* I digestion to study the dynamics of *S. aureus* intramammary infection in dairy cows with naturally occurring subclinical mastitis caused by *S. aureus*. One aspect of the study was to determine if infusion of the quarters with large numbers of different *S. aureus* strains induced clearance, co-infection, alteration or persistence of the original subclinical mastitis.

A study by Sears *et al.* (1990) concluded that routine bacteriological screening from a single quarter milk sample had a sensitivity of only 75% for diagnosis of intramammary infection caused by *S. aureus*. Another aim of the study was to determine whether *S. aureus* strain variation was evident when more than one colony of *S. aureus* per milk sample was examined using REFP analysis.

Materials and methods

Animals

Eighteen cows with naturally occurring subclinical mastitis caused by *S. aureus* kept on two commercial dairy farms (herds X and Y) were studied initially (complete data not shown). A dominant strain of *S. aureus* was associated with each herd. These were distinguishable by REFP analysis. Two cows with subclinical mastitis caused by *S. aureus* from each of these farms were employed in the experimental challenge study. These cows were selected initially as having high individual cow somatic cell

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count (> 400,000 cells/ml milk) for 3 consecutive months, and the infected quarters were subsequently identified by individual quarter somatic cell count (> 600,000 cells/ml milk) over the same time period. Somatic cells were measured in a commercial laboratory using a Coulter Counter.

Milk sample collection and routine bacterial isolation

Individual quarter milk samples were collected by hand stripping on day −4 (pre-challenge), and on days 3, 8 and 17 post-challenge. Routine bacteriological analysis was carried out in a commercial laboratory. Initial identification of *S. aureus* was based on colony morphology and a positive coagulase test.

Bacterial isolation from milk samples for REFP analysis

Milk (3 ml) was mixed with sterile water (17 ml) and centrifuged (4500 ***g*** for 20 min). The cell pellet was resuspended in 100 μl lauryl broth, 50 μl was spread onto columbia blood agar and manitol salt agar plates and incubated for 12–18 h at 37 °C. Following initial identification of *S. aureus* (as above), 15–20 individual *S. aureus* colonies from each sample were subcultured onto columbia agar slopes.

Restriction enzyme fragmentation pattern analysis

Purification of DNA was carried out using a modification of the method described by Platt *et al.* (1996). Briefly, *S. aureus* inoculated BHI broths were centrifuged (4480 ***g*** for 10 min) and resuspended in 3 × 1-ml volumes of tris ethylene diamine tetra acetic acid (EDTA) sodium chloride buffer (TES). After centrifugation (13,800 ***g*** for 30 s) pellets were resuspended in 200 μl TES (50 mM sucrose) containing 20 μl lysostaphin (1,000 units/ml) and 100 μl lysozyme (40 mg/ml), vortexed thoroughly and incubated at 37 °C. Standard DNA isolation and purification was followed as described by Platt *et al.* (1996), samples were finally microcentrifuged (13,800 ***g*** for 10 min) and resuspended in 60 μl TE (10 mM Tris base, 1 mM disodium EDTA, pH 8·0). Restriction digestion of each sample using restriction enzyme *Hha* I (Gibco Life Technologies Limited, Paisley, UK, PA4 9RF) was carried out, with bacteriophage λ DNA digested with *Hha* I, *Kpn* I and *Pst* I as controls. Restriction fragments were electrophoresed in a 0·8% agarose gel at 25 mA overnight, stained with ethidium bromide (0·5–1·0 μg/ml) and photographed on a transilluminator at 302 nm using Polaroid film type 655. Digitisation of REFP isolates allowed gel-to-gel comparisons of *S. aureus* strains. Strains were analysed using a commercially available programme (Platt & Sullivan, 1992) with λ DNA that had been digested using restriction enzymes *Kpn* I and *Pst* I as calibration controls.

Coefficient of similarity

Restriction fingerprints were compared using a coefficient of similarity following digestion with the same restriction enzyme (Dice, 1945). The molecular weight of the control fragments was fitted to a robust modified hyperbola, from which the size of restriction fragments in adjacent tracks was estimated. Numerical values were stored for subsequent graphical output which was on a logarithmic scale; the experimental variation in fragment size did not exceed 5% (Plikaytis *et al.* 1986).

Intramammary infusion with Staphylococcus aureus

Infected quarters were challenged with 10^9 *S. aureus* suspended in 20 ml Ringers Solution (Oxoid Limited, Basingstoke, UK, RG24 8PW). Two cows (Cows 1 and 2) were challenged with the same strain of *S. aureus* isolated originally from them: the

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Table 1. *The double crossover design for intramammary challenge of four cows with two different strains of* Staphylococcus aureus, *A and B*

Cow	*Staphylococcus aureus* strain	Pre-challenge	Post challenge
1	Indigenous	A	A
2	Indigenous	B	B
3	Non-indigenous	A	B
4	Non-indigenous	B	A

indigenous strain; and two cows (Cows 3 and 4) were challenged with the strain of *S. aureus* which was not isolated originally from them but isolated from the other cow: the non-indigenous strain (Table 1).

Throughout the study, uninfected quarters designated as control quarters were infused with 20 ml Ringers Solution. These quarters were examined to confirm that cross-contamination had not occurred during the intramammary infusion procedure and subsequent sampling.

Teats were thoroughly disinfected with cotton wool soaked with 70% alcohol immediately prior to intramammary infusion performed using a 6FG canine urinary catheter. Following infusion with bacteria, the quarters were massaged in an upward direction, and all teats were dipped in an iodophor teat dip (Superteat, Alfa Laval Agri, Oakfield, Gwent, UK). The cows were examined clinically at regular intervals for 48 h after the infusion procedure and were milked twice daily by machine.

RESULTS

Routine isolation of Staphylococcus aureus *from milk samples*

Quarters were recorded as either *S. aureus* positive or *S. aureus* negative and the presence of other bacterial species isolated on culture was also recorded. Quarters identified as *S. aureus* negative pre-challenge and designated as control quarters, remained *S. aureus* negative on days 3, 8 and 17 post-challenge in all animals and demonstrated that cross contamination of quarters with *S. aureus* had not occurred. Three of the four cows, Cows 1, 3 and 4, were identified as *S. aureus* positive following routine bacteriological analysis at all sampling points pre- and post-challenge. However, Cow 2 was identified as *S. aureus* positive following routine bacteriological analysis on day –4 (pre-challenge) and on days 3 and 8 post-challenge, but by day 9 post-challenge this animal had developed severe mastitis including reddening and swelling of the quarter. The cow was pyrexic and anorexic and was subsequently removed from the study and given systemic anti-inflammatory treatment and intramammary antibiotics.

Recognition of different Staphylococcus aureus *strains*

Figure 1 illustrates a range of REFPs from bovine *S. aureus* from herds X and Y. Of the 18 isolates, 10 showed identical REFPs (lanes 3, 4, 7, 9, 11–13, 16, 18, 19), the dominant strain in herd X. Two isolates (lanes 6 and 8) show two band differences (differ in a single fragment) and are molecular variants of the dominant strain. Five identical isolates showed 10 band differences and were regarded as distinct from the dominant strain. The single strain from herd Y showed > 10 band differences from the dominant strain (lane 14).

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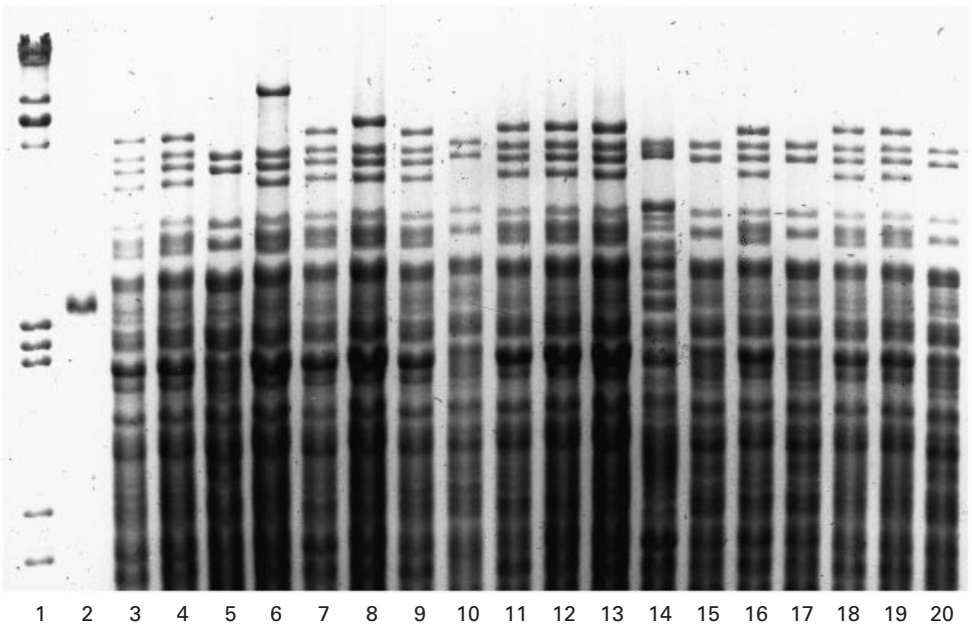

Fig. 1. Restriction enzyme fragmentation pattern (REFP) of bacterial isolates after digestion with *Hha* I, ethidium bromide (0·5–1·0 μg/ml) stained 0·8% agarose gel electrophoresis. Lanes 3–13 and 15–20 show isolates from herd X. Lane 14 shows a single isolate from a geographically distinct herd (Y). Bacteriophage λ DNA controls were digested with *Hha* I, *Kpn* I and *Pst* I. Lanes 1 and 2 show *Kpn* I and *Pst* I respectively.

Table 2. *Restriction enzyme fragmentation pattern (REFP) of bacterial isolates from cows 1, 2, 3 and 4 identified in subclinically infected and challenged quarters. A = Strain A; B = Strain B; R_x = additional isolate; ND = No REFP analysis possible, removed from study on day 9 post-challenge*

		Post challenge	Post challenge	Post challenge
	Pre-challenge	Day 3	Day 8	Day 17
Cow 1	AAAAA	AAAAA	AAAAA	AAAAA
Indigenous (A+A)	AAAAA	AAAAA	AAAAA	R_2
Cow 2	BBBBB	BBBBB	R_3 R_4	ND
Indigenous (B+B)	BBBBB	BBBBB	R_6 R_7	
Cow 3	AAAAA	AAAAA	AAAAA	AAAAA
Non-indigenous (A+B)	AAAAA	AAAAA	AAAAA	AAAAA
Cow 4	BBBBB	AAAAA	BBBBB	BBBBB
Non-indigenous (B+A)	BBBBB	AAAAA	BBBBB	BBBBB

Restriction enzyme fragmentation pattern analysis

Bacterial isolates from all four quarters of each cow in the experimental challenge study were fingerprinted and analysed by REFP on four occasions during the study i.e. day −4 (pre-challenge) and days 3, 8, and 17 (except Cow 2) post-challenge (Table 2). A total of 400 isolates were studied by REFP analysis.

Approximately 63% of the isolates tested were characterised following digestion

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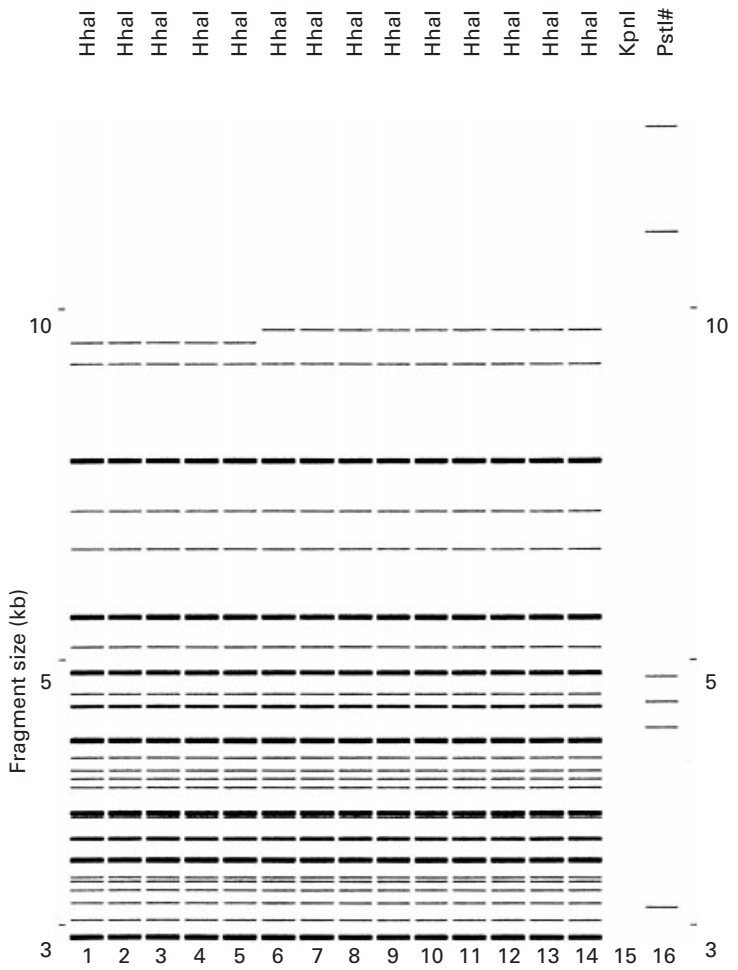

Fig. 2. Graphical output of digitised image of REFP of bacterial isolates after digestion with *Hha* I; ethidium bromide (0·5–1·0 μg/ml) stained 0·8% agarose gel electrophoresis. Lanes 1–5 show isolates identified as strain A*. Lanes 6–14 show isolates identified as strain A. Bacteriophage λ DNA controls were digested with *Hha* I, *Kpn* I and *Pst* I. Lanes 15 and 16 show *Kpn* I and *Pst* I respectively.

of bacterial DNA with *Hha* I as a strain with 25 fragments ranging from 2·92 kb to 9·59 kb in size, designated strain A (shown in digitised form in Fig. 2, Lanes 6–14). Approximately 10% of the isolates tested were identified as a molecular variant of strain A, designated as A*. After digestion of strain A* with *Hha* I, 24 of the 25 fragments match those of strain A (shown in digitised form in Fig. 2, Lanes 1–5). Only the largest fragment differed between strain A and strain A* (a 9·59-kb fragment in strain A and a 9·32-kb fragment in strain A*). Approximately 22% of the isolates were identified following digestion of bacterial DNA with restriction digest *Hha* I, as a strain with 30 fragments with a size range of 2·92–9·59 kb, this was designated strain B (shown in digitised form in Fig. 3, Lanes 3, 4, 6, 8–15). The remaining 4% of the isolates tested comprised of seven genetically unrelated strains which were recognised by REFP analysis as strains other than strain A, strain A* or strain B and were designated strains R_1 to R_7 (shown in digitised form in Fig. 4, Lanes 5–11).

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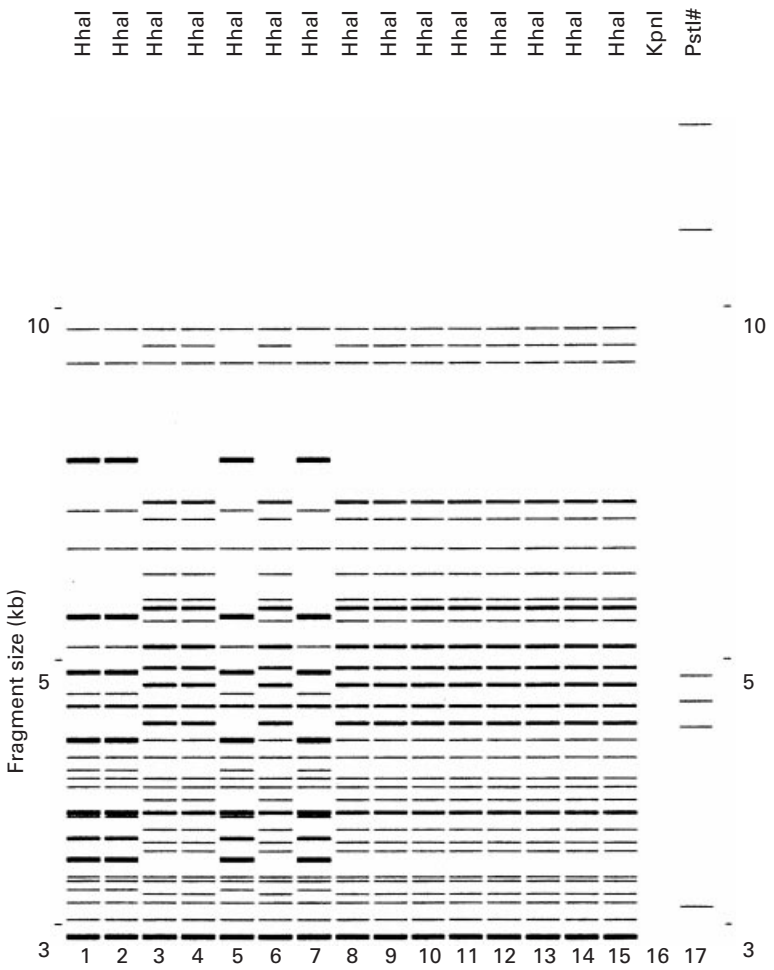

Fig. 3. Graphical output of digitised image of REFP of bacterial isolates after digestion with *Hha* I; ethidium bromide (0·5–1·0 μg/ml) stained 0·8% agarose gel electrophoresis. Lanes 1, 2, 5 and 7 show strain A, and lanes 3, 4, 6, 8–15 show strain B. Bacteriophage λ DNA controls were digested with *Hha* I, *Kpn* I and *Pst* I. Lanes 15 and 16 show *Kpn* I and *Pst* I respectively.

Coefficient of similarity among strains

The coefficient of similarity (S_D) between strain A and strain A* was 96% (24 of the 25 fragments matching), between strain A and strain B, 83·6% (23 of the 30 fragments matching). S_D between strain A* and strain B 83·6% (23 of the 30 fragments matching). Strain A and strain A* had between 48·9–73·7% similarity to strains R_1 to R_7, whereas strain B had between 52·0–83·9% similarity to strains R_1 to R_7 (Table 3).

Cows challenged with the indigenous strain of Staphylococcus aureus

Cow 1 was subclinically infected with strain A and challenged with strain A (Table 1). REFP analysis of ten colonies of pre-infusion isolates confirmed the presence of a homogeneous population of strain A. REFP analysis of ten colonies of post-challenge isolates confirmed strain A as a homogeneous population on days 3 and 8, but on day 17, five colonies were characterised as strain A while only a single

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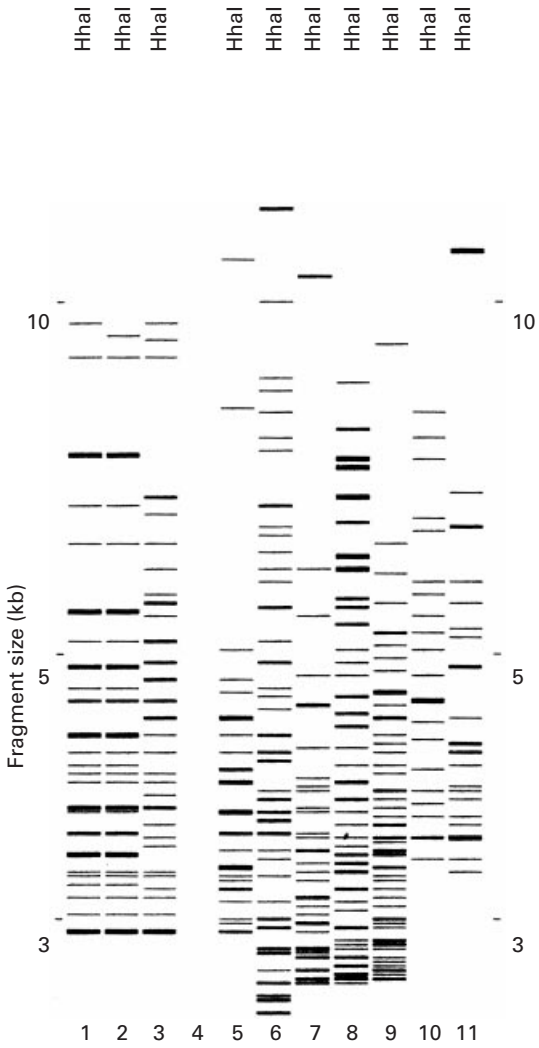

Fig. 4. Graphical output of digitised image of REFP of bacterial isolates after digestion with *Hha* I; ethidium bromide (0·5–1·0 μg/ml) stained 0·8% agarose gel electrophoresis. Lane 1 shows strain A, lane 2 shows strain A*, lane 3 shows strain B and additional distinct isolates R_1–R_7 are shown in lanes 5–11 respectively.

Table 3. *Coefficient of similarity between strains, identified by REFP analysis and analysed following digitisation*

Isolate	Total no. of fragments analysed	Range of fragment sizes (kilobases)	Similarity with strain A (%)	Similarity with strain B (%)
A	25	2·92– 9·59	—	83·6
B	30	2·92– 9·59	83·6	—
A*	25	2·92– 9·32	96·0	83·6
R_1	22	2·93–15·88	63·8	61·5
R_2	33	2·95–12·01	72·4	66·7
R_3	22	2·92–10·49	68·1	69·2
R_4	33	2·89– 8·52	73·7	83·9
R_5	36	2·86– 9·19	71·2	75·0
R_6	20	3·38– 8·06	53·3	52·0
R_7	20	3·30–10·99	48·9	64·0

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isolate was characterised as R_2 (Table 2). Cow 2 was subclinically infected with strain B and challenged with strain B (Table 1). REFP analysis of 10 colonies of pre-infusion isolates confirmed the presence of a homogeneous population of strain B. REFP analysis of 10 colonies of post-challenge isolates confirmed strain B as a homogeneous population on day 3. Single isolates designated R_3, R_4, R_6 and R_7 were identified as a mixed population in the quarter on day 8 (Table 2).

Cows challenged with the non-indigenous strain of Staphylococcus aureus

Cow 3 was subclinically infected with strain A and challenged with strain B. REFP analysis of ten colonies of pre-infusion isolates confirmed the presence of a homogeneous population of strain A. REFP analysis of ten colonies of post-challenge isolates confirmed the presence of a homogeneous population of strain A in the quarter throughout the duration of the experiment, on days 3, 8 and 17 (Table 2). Cow 4 was subclinically infected with strain B and challenged with strain A. REFP analysis of ten colonies of pre-infusion isolates confirmed the presence of strain B as a homogeneous population. REFP analysis of ten colonies of post-challenge isolates identified strain A, the challenge strain, as a homogeneous population on day 3. By day 8 post-challenge REFP analysis of ten colonies identified that strain B had, once again, become a homogeneous population, and remained the sole isolate to day 17 post-challenge when sampling ended (Table 2).

DISCUSSION

Previous studies to characterise *S. aureus* isolates in European dairy herds has shown evidence of clonal variation within individual herds (Lam *et al.* 1996; Annemuller *et al.* 1999). This observation was based on the analysis of a single colony from each sample tested as in standard diagnostic procedures. This raised the important question of whether strain variation reflected the choice of colony for study, genotypic diversification within the herd, or whether individual cows harboured one, or more, variants of *S. aureus*. Furthermore, the chronicity of bovine *S. aureus* mastitis raises the possibility that cows may acquire different strains of *S. aureus* that remain co-resident within the udder and which may potentially suppress the host immune response and allow infection to persist. Thus, one of the initial aims of this study was to determine whether, and to what extent, strain variation was evident within an individual quarter or cow.

In the present study, routine bacteriological investigation in a commercial laboratory resulted in *S. aureus* being isolated from the challenged quarters on all occasions in three of the four cows (1, 3 and 4). REFP analysis based on restriction enzymes that recognise a 4-base rather than 6-base cleavage site in DNA was used to provide a large amount of information, amenable to computer analysis and adaptable to a wide range of micro-organisms (Platt *et al.* 1996). From previous case-control studies of *S. aureus* infection, variation in 1–3 restriction fragments constituted clonal variation and unrelated strains differed in seven or more restriction fragments (Brown, 1998).

REFP analysis allowed discrimination of two clonal variants of the same strain of *S. aureus*, strain A and A* in this study. Strain A and A* had almost identical REFP patterns that differed by a single restriction fragment (clonal variants). REFP also discriminated between the unrelated strains A and B, the main strains involved in the study, and strains R_1 to R_7 which were identified infrequently. These findings were similar to those of Jayarao *et al.* (1991) who used REFP analysis

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to identify and differentiate closely related and unrelated strains of another mastitis pathogen, *Streptococcus uberis*, which had been isolated from bovine mammary secretions.

Multiple colony analysis in this project showed that, on several occasions, more than one strain of *S. aureus* was isolated from a single quarter. Both related and unrelated strains of *S. aureus* were isolated from the same quarter on the same sampling day in an individual animal, as also reported for *S. uberis* by Jayarao *et al.* (1991). Multiple colony analysis is, therefore, a useful technique in detailed studies of bovine mastitis caused by *S. aureus*.

By comparing isolates between and within herds, it is possible to draw conclusions about the source and spread of bacteria. For example, the study by Lam *et al.* (1996) showed that different *Escherichia coli* genotypes were identified from different quarters, suggesting that *E. coli* is an environmental pathogen and does not spread from quarter to quarter. Whereas among *S. aureus* a limited number of genotypes (11) were identified among 63 isolates from five herds, supporting the theory that contagious pathogens transfer between cows and quarters within a herd. The observation that herd X in the present study harboured a dominant strain and fewer isolates of a sub-dominant strain that differed genotypically supports this view.

Results from the present study also indicate the existence of several strains of *S. aureus* identified from the four experimentally challenged cows studied. REFP analysis identified eight additional strains other than the two main strains in the study, strain A and strain B, and demonstrated that mixed infection occurs and that the investigation of a single colony may not provide a complete understanding of the epidemiology of intramammary infection.

The recognition of molecular variants of *S. aureus* is not surprising given the chronic nature of bovine staphylococcal mastitis and this may reflect the potential for genetic change over extended time periods. It also raises questions concerning the nature of selection pressure in both individual animals and the farm environment in general and requires further investigation.

The double crossover intramammary challenge was employed to determine if the indigenous strain was displaced by the non-indigenous strain or whether the indigenous strain persisted. Another aim was to show whether superinfection with the indigenous strain altered, possibly aiding clearance of infection by inducing a secondary immune response to the specific bacterial strain, or had no effect on the original infection status of the infected quarter.

Intramammary infection persisted throughout the study in the two cows challenged with the indigenous strain of *S. aureus*. This suggests that challenge with a high dose of the indigenous strain of *S. aureus* did not induce a secondary immune response which may have resulted in subsequent clearance of the persistent infection. However Cow 2 developed more severe clinical signs of mastitis on day 9 post-challenge and four different strains, R_3, R_4, R_6, and R_7 (that differed from the indigenous strain B) were identified on day 8 post-challenge. It is possible, therefore, that challenge with indigenous strain B induced an immune response that may have allowed re-emergence of other previously persistent strains within the mammary gland. As the cow required treatment and was withdrawn from the study, it was not possible to isolate strains subsequent to the clinical episode.

Two cows were challenged with the non-indigenous strain of *S. aureus*. This part of the study showed that when infected with the indigenous strain (either strain A or strain B) and subsequently challenged with the non-indigenous strain, the indigenous strain persisted as the dominant strain within the quarter and was the

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only strain identified on day 17 post-challenge in both cows. This study suggests that intramammary challenge of subclinically infected quarters with the non-indigenous strain did not result in permanent replacement of the indigenous strain with the recently infused non-indigenous strain. This may reflect hyporesponsiveness of mammary gland immunity that may be innate or induced as a result of chronic infection and inflammation. Failure of establishment of the challenge non-indigenous strain may have resulted from suppression of immune responses by the resident bacteria or competition among strains for physiological or pathological requirements in the mammary tissues. The persistent subclinical infection in three of the four animals confirms the chronicity of *S. aureus* intramammary infection which has been well documented in field and in experimental studies (Knight *et al.* 2000).

In contrast to the study by Sears *et al.* (1990) that employed a low challenge dose (20–2,000 CFU/ml) of virulent *S. aureus* by the intramammary route and resulted in infection of 19 of 21 previously uninfected glands, in the present study, the strains selected were isolated from cows with subclinical mastitis which are presumably less virulent than strains involved in acute clinical mastitis. It was considered that it was necessary to infuse large numbers of bacteria into an infected gland in this study in order to provide a significant challenge to the substantial numbers of organisms already established in the gland.

Further work is required to develop a rapid identification system to determine *S. aureus* strain diversity as an epidemiological tool for future mastitis investigations. The persistence of subclinical *S. aureus* mastitis in the cows in this study highlights the importance of improved understanding of the bovine udder immune system and its defence against *S. aureus* and other major mastitis pathogens.

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