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Fine-structure molecular epidemiological analysis of *Staphylococcus aureus* recovered from cows

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SUMMARY

Sixty-three *Staphylococcus aureus* isolates recovered from bovine sources in the USA and the Republic of Ireland were characterized by multilocus enzyme electrophoresis (MLEE), ribotyping, and random amplified polymorphic DNA polymerase chain reaction (RAPD–PCR) typing at two separate laboratories. The *S. aureus* isolates were assigned by MLEE to 10 electrophoretic types (ETs) (Index of Discrimination, $D = 0{\cdot}779$). In contrast, the same isolates were assigned to 13 ribotypes ($D = 0{\cdot}888$), and to 12 RAPD types ($D = 0{\cdot}898$). A common clone, ET3, of worldwide distribution, was represented by six distinct combinations of ribotypes and RAPD types. *S. aureus* clones recovered from cows in Ireland were also associated with mastitis in dairy cows in the USA. These findings are consistent with the hypothesis that only a few specialized clones of *S. aureus* are responsible for the majority of cases of bovine mastitis, and that these clones have a broad geographic distribution.

INTRODUCTION

Staphylococcus aureus is a major pathogen in chronic bovine mastitis, causing severe financial losses worldwide [1]. Studies on the genetic diversity and structure of natural populations of a number of human and animal pathogens have revealed that (i) most species are clonal in nature, (ii) the number of clones is relatively small, and (iii) the majority of cases of serious disease is caused by a small proportion of the total number of existent clones [2, 3]. Studies on natural populations of *S. aureus* have identified considerable genetic heterogeneity [3–6]. Thus, the effective control of mastitic infections caused by staphylococci may have to involve directed and rational strategies against clones of *S. aureus* commonly found in diseased udders.

Numerous methods have been utilized for discrimination and comparison of *S. aureus* isolates in epidemiological investigations of staphylococcal infections. More traditional biochemical and physiological typing methods, such as bacteriophage typing, biotyping, antibiotic sensitivity testing, toxin and enzyme profiling, and plasmid screening, have been superseded in the past decade by a plethora of molecular genetic procedures, such as ribotyping, plasmid DNA restriction patterns, pulsed-field gel electrophoresis of macrorestriction DNA fragments, and random amplified polymorphic DNA (RAPD) polymerase chain reaction (PCR) analysis, [4, 6–11]. These molecular techniques have proved useful for grouping isolates

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into convenient intraspecies subsets, which has been of particular value in the monitoring of nosocomial spread of staphylococci [6] and in the clonal analysis of methicillin-resistant staphylococci [12, 13].

Multilocus enzyme electrophoresis (MLEE) analysis is a molecular technique which has been extensively employed in bacterial population genetics research [14]. It indexes allelic variation in sets of randomly selected genes of the bacterial chromosome, thereby providing a means for estimating overall levels of single-locus and multilocus genotypic variation within a species. Electromorphs (mobility variants) of an enzyme can be directly equated with alleles of the corresponding gene, and electromorph profiles of different enzymes (electrophoretic types or ETs) correspond to multilocus chromosomal genotypes. MLEE analysis of isolates of *S. aureus* has demonstrated a predominantly clonal structure [15]. A recent MLEE study of 357 *S. aureus* isolates recovered from milk showed that the great majority (nearly 90%) of these was represented by only eight clonal ETs [16]. Moreover, a large proportion (28·9%) of these bovine isolates from worldwide sources was grouped into a single clonal type, designated ET3.

RAPD–PCR [16–18] has been applied successfully to the genetic fingerprinting of many bacterial species including *S. aureus* [4, 6, 19, 20]. It is both rapid and discriminatory. Ribotyping, the use of rRNA-based probes for typing [8], has emerged as one of the most commonly utilized molecular epidemiological methods [4, 5, 24, 25].

The present study was carried out to determine the genetic relationships among *S. aureus* isolates associated with bovine mastitis in the USA and Ireland. This investigation applies the DNA-based fingerprinting techniques RAPD–PCR and ribotyping in combination with MLEE to enable the construction of a 'fine-structure', molecular genetic framework of the *S. aureus* population isolated from bovine mastitis.

MATERIALS AND METHODS

Bacterial isolates, media and growth conditions

Twenty-one *S. aureus* isolates were taken from dairy cattle with intramammary infection in the south-west region of Ireland. They were isolated on 7% (v/v) calf blood tryptic soy agar (TSA, Difco) and identified on the basis of Gram stain, coagulase activity and acetoin production. The 21 *S. aureus* isolates recovered from cows in Ireland were chosen as being representative of the three major RAPD types found in a study of over 150 Irish isolates from the mammary glands of cows (data not shown) with the following isolation frequencies: RAPD type 4, 24·5%; RAPD type 5, 50%; and RAPD type 7, 21%. Four of the selected Irish isolates belonged to RAPD type 4, 8 to RAPD type 5, and 9 to RAPD type 7 (Table 1). Initially, only three RAPD type 7 isolates were chosen at random, reflecting the proportional distribution of this RAPD type. However, subsequent phenotyping of RAPD type 7 isolates revealed that these three isolates did not produce enterotoxin C (EntC) and toxic shock syndrome toxin-1 (TSST-1), whereas approximately 75% of RAPD type 7 isolates were $EntC^{+}TSST\text{-}1^{+}$. Accordingly, a further six $EntC^{+}TSST\text{-}1^{+}$ isolates of RAPD type 7 were included in the study (Table 1).

The 42 *S. aureus* isolates recovered from cows in the USA were chosen as being representative of the 8 predominant ETs identified using MLEE in a previous study of 357 isolates [15], in which they accounted for the following proportions: ET1, 6·7%; ET2, 2·2%, ET3, 28·9%; ET5, 17·9%; ET6, 2%; ET7, 12·7%; ET36, 14·6%; and ET39, 4·2%. Six of the selected *S. aureus* isolates recovered from cows in the USA belonged to ET1, 5 to ET2, 9 to ET3, 5 to ET5, 4 to ET6, 5 to ET7, 5 to ET36, and 3 to ET39 (Table 1). The numbers of isolates belonging to ETs 3, 5, 7 and 36 reflected the relative frequencies of their occurrence in the study population of Kapur and colleagues [15]. If the numbers of isolates of ETs 1, 2, 6 and 39 had been selected on a relative frequency basis, only one or two isolates of each would have been analysed in a study of this size, so equivalent numbers of isolates to the other ETs, except ET3, were examined.

S. aureus bacteria were grown on 7% (v/v) sheep blood TSA at 37 °C overnight. Isolates were stored in tryptic soy broth (TSB) containing 15% (v/v) glycerol at −70 °C. Bacteria were grown in TSB at 37 °C overnight with constant shaking at 150 r.p.m.

Preparation of genomic DNA

Of a TSB overnight culture, the bacteria from 1 ml were pelleted and washed in 200 μl TE buffer (10 mM-Tris-HCl containing 1 mM-EDTA, pH 7·5). The cells were resuspended in 50 ml 25% (w/v) sucrose in 50 mM-Tris-HCl buffer, pH 8·0 and placed on ice. Then, 10 μl lysostaphin (Sigma, 1 mg/ml in 50 mM-Tris-HCl buffer, pH 8·0) was added and the mixture was incubated at 37 °C for 20 min in a waterbath. Next, 20 μl proteinase K (Boehringer, 20 mg/ml in

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Table 1. *Typing of bovine* S. aureus *isolates*

Isolate	Origin	Herd	ET†	RAPD type	Ribo-type
10·1	USA		1	6	8
13·1	USA		1	6	10
930	USA		1	10	6
1005	USA		1	10	6
1047	USA		1	10	6
916	USA		1	12	6
103·14	USA		2	2	2
10·6	USA		2	2	9
72·3	USA		2	2	9
1460	USA		2	2	9
1003	USA		2	11	5
1014	USA		3	4	11
1058	USA		3	4	11
111	IRL	Mitchelstown	3	4	11
4·2	USA		3	4	12
115	IRL	Mallow	3	4	12
116	IRL	Mallow	3	4	12
539	USA		3	5	7
1369	USA		3	5	7
101	IRL	Tipperary	3	5	7
103	IRL	Ballyderown	3	5	7
104	IRL	Ballyderown	3	5	7
105	IRL	Ballyderown	3	5	7
106	IRL	Ballyderown	3	5	7
107	IRL	Ballyderown	3	5	7
118	IRL	Moorepark	3	5	7
123	IRL	Mallow	3	5	7
1–2	USA		3	8	4
32	USA		3	8	4
1–1	USA		3	8	4
919	USA		3	8	12
102*	IRL	Tipperary	3	7	4
108*	IRL	Ballyderown	3	7	4
110*	IRL	Tipperary	3	7	4
113*	IRL	Tipperary	3	7	4
119*	IRL	Unknown	3	7	4
120	IRL	Aghada	3	7	4
121	IRL	Aghada	3	7	4
122*	IRL	Ballymacoda	3	7	4
17·1	USA		5	3	9
927	USA		5	4	12
1011	USA		5	4	12
3	USA		5	8	4
1547	USA		5	11	5
932	USA		6	1	3
1468	USA		6	1	3
1534	USA		6	1	3
1378	USA		6	8	4
1455	USA		7	1	5
1521	USA		7	1	5
20·1	USA		7	1	5
77	USA		7	1	5
1363	USA		7	1	13
26·1	USA		36	9	1
73·2	USA		36	9	1
12–1	USA		36	9	1
5	USA		36	9	1
148	USA		36	9	1
6–1	USA		39	9	1
31	USA		39	9	1
37	USA		39	9	1
114	IRL	Cork	40	7	4
117	IRL	Mallow	41	4	12

* RAPD type 7 isolates producing EntC and TSST-1.
† ET, Electrophoretic type.

10 mM-Tris-HCl buffer, pH 8·0) was added, and the mixture incubated at 50 °C for 1 h, followed by addition of 20 μl 0·5 M-EDTA and then 20 μl 10% (w/v) sodium *N*-lauroyl sarcosine (Sigma). This lysate was mixed gently and kept on ice for 1 h. The mixture was then incubated for 1 h at 55 °C. TE (400 μl) was added, followed by 100 μl 5 M-NaCl. This was mixed thoroughly before addition of 80 μl 10% (w/v) cetyltrimethylammoniumbromide (CTAB) in 0·7 M-NaCl and incubation at 65 °C for 10 min. The mixture was extracted with chloroform:isoamyl alcohol (24:1) and then with phenol:chloroform:isoamyl alcohol (25:24:1), followed by precipitation of nucleic acid with 0·6 vol isopropanol. The DNA was resuspended in an appropriate volume of TE.

RAPD–PCR analysis of bacterial isolates

RAPD–PCR was carried out as described previously [25]. The cycling programme was 4 cycles of [94 °C, 5 min; 36 °C, 5 min; and 72 °C, 5 min], 30 cycles of [94 °C, 1 min; 36 °C, 1 min; and 72 °C, 2 min], and then 72 °C, 10 min. The 23 nucleotide-long primer (D11344) used in these PCR reactions was 5′-AGTGAATTCGCGGTGAGATGCCA-3′ [25].

Ribotyping

Approximately 5 μg *S. aureus* DNA was digested overnight at 37 °C with restriction endonuclease *Hin*dIII, which was used according to the manufacturer's recommendations (Boehringer). Restriction fragments were separated by conventional electrophoresis (2 V/cm) on 0·8% (w/v) agarose gel in 0·5 × TBE, and transferred onto an Immobilon P

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membrane (Millipore) by the method of Southern [26]. Prehybridization was performed with 6 × SSC (1 × SSC is 0·15 M-NaCl, 0·015 M-sodium citrate), 5 × Denhardt's solution, 50 mg salmon sperm DNA/ml (Sigma) and 0·1 % (w/v) sodium dodecyl sulphate (SDS) at 65 °C for 2 h.

Escherichia coli 16S and 23S RNA was labelled with [α-^{32}P]ATP (Amersham) using the Prime-a-Gene kit (Promega). The hybridization between staphylococcal DNA and *E. coli* rRNA was performed overnight at 65 °C in prehybridization solution, supplemented with $2{\cdot}5 \times 10^6$ d.p.m. (50 ng) of labelled rRNA per 20 ml and per membrane. After the hybridization step, the filters were washed twice for 10 min in 2 × SSC containing 0·1 % (w/v) SDS at 50 °C and twice for 10 min in 0·1 × SSC containing 0·1 % (w/v) SDS at 30 °C. Hybridized bands were visualized by autoradiography with Cronex 10 s film using two Quanta Rapid intensifying screens (Dupont) for between 8 and 48 h at −70 °C. A 100 bp DNA ladder and bacteriophage λ DNA digested with restriction endonuclease *Hin*dIII were used as size standards on all gels.

MLEE analysis of bacterial isolates

MLEE was carried out as described elsewhere [14, 16]. Following electrophoresis on starch gels, selective histochemical staining for 13 metabolic enzymes [aconitase, carbamylate kinase, mannitol-1-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, nucleoside phosphorylase, catalase, α,β-naphthyl propionate esterase, L-lactate dehydrogenase, alcohol dehydrogenase, shikimic acid dehydrogenase, glucose-6-phosphate dehydrogenase, glutamate dehydrogenase (NAD-dependent) and phosphoglucose isomerase] was conducted. Each isolate was characterized by its combination of alleles at 13 enzyme loci. Unique combinations of electromorphs were designated as electrophoretic types (ETs) [5, 14, 16].

Discriminatory power, concordance analysis and statistical analysis

To compare the discriminatory power of the typing methods, the index of discrimination (D) as proposed by Hunter [27] was applied. Concordance analysis [28] of MLEE, RAPD–PCR and ribotyping was carried out as previously described. The G-test of independence [29] was used to assess statistical significance.

RESULTS

RAPD–PCR

RAPD typing subdivided the 63 isolates into 12 fingerprint groups (Fig. 1). Subdivision was based on the characteristic patterns of PCR amplimers between 400 and 1500 bp (Fig. 1). Amplimers of larger size were not always clearly resolved and appeared similar in size distribution. Of these 63 isolates, 30 were re-examined by RAPD–PCR using new DNA preparations as templates, to assess reproducibility. The resulting profiles were indistinguishable from those originally determined. The RAPD types could be divided into two sub-groups on the basis of the presence of distinctive amplimer patterns in the 600–800 bp range (RAPD types 6–12) and the presence of a distinctive amplimer pattern in the 600–1000 bp range (RAPD types 1–5). Distinguishing features of RAPD amplimer patterns are identified in Figure 1.

In the first subgroup, RAPD types 6–8 were characterized by the presence of prominent amplimers of 680 bp and 740 bp. These RAPD types were distinguished by the following features: the absence of an amplimer of 980 bp in RAPD type 6, the absence of an amplimer of 540 bp in RAPD type 8 and the presence of an amplimer of 780 bp in RAPD type 6. RAPD type 9 differed from RAPD types 6–8 by the absence of the prominent amplimer of 680 bp and the presence of a prominent amplimer of 540 bp. RAPD type 10 had a prominent amplimer of 640 bp, lacked the 680 bp amplimer and possessed a weaker amplimer of 740 bp. RAPD type 11 had weak amplimers of 680 bp and 740 bp and possessed two amplimers of 890 bp and 910 bp. RAPD type 12 had a prominent amplimer of 740 bp. This RAPD type was similar to RAPD type 6 in lacking an amplimer of 980 bp but differed by the absence of an amplimer of 540 bp and the presence of a less prominent amplimer of 680 bp.

In the second subgroup, RAPD type 1 differed by the presence of amplimers of 700 bp and 960 bp. RAPD type 2 differed from all other RAPD types by the presence of a prominent amplimer of 890 bp and from types 3, 4 and 5 by the absence of an amplimer of 850 bp. RAPD type 4 differed from RAPD type 3 by the presence of weak amplimers of 940 bp and 960 bp. RAPD type 5 differed from RAPD types 3 and 4 by the presence of a weaker amplimer of 1,080 bp and the absence of an amplimer of 590 bp.

The 21 *S. aureus* isolates recovered from cows in Ireland were representative of three fingerprint

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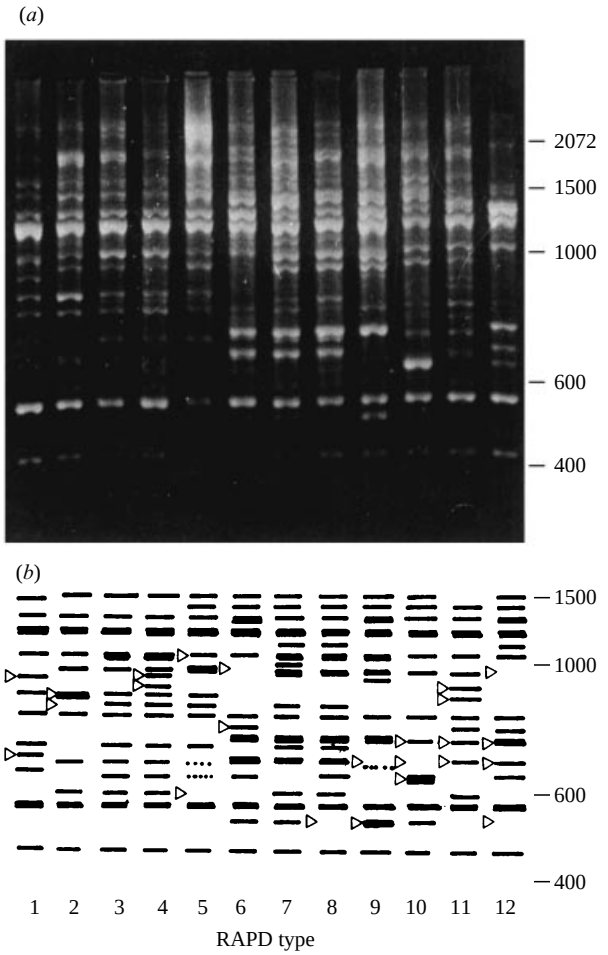

Fig. 1. A: Agarose gel electrophoresis of RAPD–PCR products representative of the 12 fingerprint types produced by 63 isolates of *S. aureus* from bovine mastitis. RAPD–PCR was performed on genomic DNA using 23 oligonucleotide-long primer. RAPD type 1, strain 20·1 (ET7); RAPD type 2, strain 10·6 (ET2); RAPD type 3, strain 17·1 (ET5); RAPD type 4, strain 115 (ET3); RAPD type 5, strain 105 (ET3); RAPD type 6, strain 10·1 (ET1); RAPD type 7, strain 108 (ET3); RAPD type 8, strain 1–2 (ET3); RAPD type 9, strain 5 (ET36); RAPD type 10, strain 930 (ET1); RAPD type 11, strain 1547 (ET5); RAPD type 12, strain 916 (ET1). Positions of molecular size reference markers in basepairs are indicated on the right of the panel. B: Schematic diagram of RAPD fingerprint patterns. Positions of distinctive features used in pattern discrimination are marked by arrows.

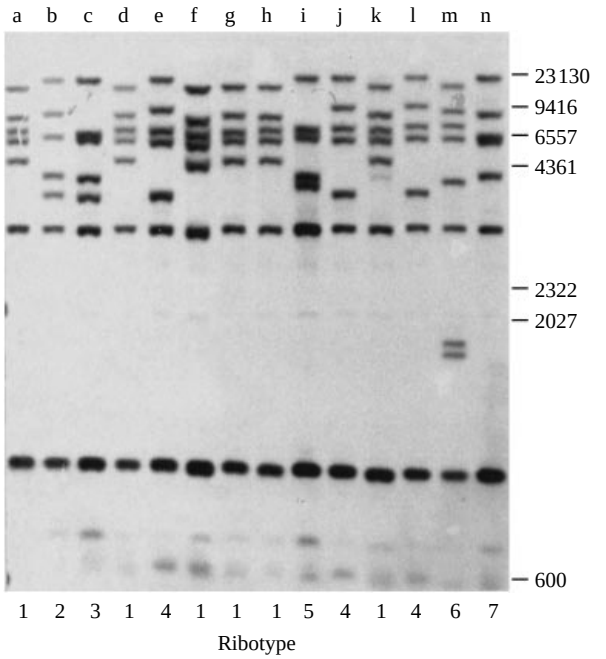

Fig. 2. rRNA gene hybridization band patterns of *S. aureus* genomic DNA digested with restriction endonuclease *Hin*dIII. Profiles produced by 14 isolates are shown. Each ribotype is assigned a number at the bottom of each lane. Lane a, strain 37 (ET39); lane b, strain 103·14 (ET2); lane c, strain 1534 (ET6); lane d, strain 26·1 (ET36); lane e, strain 3 (ET5); lane f, strain 31 (ET36); lane g, strain 148 (ET36); lane h, strain 6–1 (ET39); lane i, strain 77 (ET7); lane j, strain 1–2 (ET3); lane k, strain 73·2 (ET39); lane l, strain 1–1 (ET3); lane m, strain 916 (ET1); lane n, strain 593 (ET3). Positions of molecular size reference markers in base pairs are shown on the right of the panel.

groups, two of which were found among the isolates recovered from cows in the USA. RAPD type 7 was unique to isolates recovered from cows in Ireland. The *S. aureus* isolates recovered from cows in the USA yielded 11 RAPD types. Eight of the 63 isolates belonged to RAPD type 1, 4 to RAPD type 2, 1 to RAPD type 3, 9 to RAPD type 4, 10 to RAPD type 5, 2 to RAPD type 6, 9 to RAPD type 7, 6 to RAPD type 8, 8 to RAPD type 9, 3 to RAPD type 10, 2 to RAPD type 11, and 1 to RAPD type 12 (Table 1). RAPD typing had the greatest discriminatory power (D) of 0·898.

Ribotyping

Of the 63 isolates ribotyped, 10 were repeated, and each gave RFLP profiles which were identical to those originally determined. The 63 isolates could be grouped into 13 ribotypes. The 21 *S. aureus* isolates recovered from cows in Ireland belonged to 4 ribotypes. Each of these ribotypes was represented among the isolates recovered from cows in the USA. Representative hybridization band patterns for 14 isolates belonging to 7 ribotypes are shown in Figure 2. Of the 63 isolates, 8 belonged to ribotype 1, 1 to ribotype 2, 3 to ribotype 3, 14 to ribotype 4, 6 to ribotype 5, 4 to ribotype 6, 10 to ribotype 7, 1 to ribotype 8, 4 to ribotype 9, 1 to ribotype 10, 3 to

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Table 2. *Concordance analyses of ET with RAPD type and ribotype*

	No. of isolate pairs (proportion of total comparisons) with			
	RAPD type		Ribotype	
ET	Match	Mismatch	Match	Mismatch
Same	131 (0·07)	311 (0·16)	141 (0·07)	301 (0·15)
Different	68 (0·04)	1443 (0·74)	80 (0·04)	1431 (0·73)
Concordance*	80·6%		80·5%	

* Concordance equals the sum of the same ET-match and different ET-mismatch entries, expressed as a percentage of the total 1953 pairwise comparisons.

ribotype 11, 7 to ribotype 12 and 1 to ribotype 13. Ribotyping had a discriminatory power (D) of 0·888.

MLEE

The 63 isolates were subdivided into 10 ETs (Table 1). Of these isolates, 28 belonged to the main ET type 3. Of the 21 *S. aureus* isolates recovered from cows in Ireland, 19 belonged to ET3, and the other 2 each produced unique ETs. MLEE had the lowest discriminatory power (D) of 0·779.

Combinations of ETs, RAPD types and ribotypes

The 28 ET3 isolates were subdivided into 4 RAPD types. All ET7 isolates were of the same RAPD type 1 and similarly, all ET36 and ET39 isolates were of RAPD type 9. In addition, some of the RAPD types shared by multiple isolates were unique to certain ETs, although not all isolates within these ETs were of the same RAPD type, namely, RAPD type 2 to ET2, RAPD type 5 to ET3, RAPD type 6 to ET1, and RAPD type 10 to ET1. RAPD typing in combination with MLEE analysis subdivided 5 of the 10 ETs.

The 28 ET3 isolates were subdivided into 4 ribotypes. All 8 isolates of either ET36 or ET39 belonged to ribotype 1. In addition, some ribotypes shared by multiple isolates were unique to certain ETs, although not all isolates within these ETs were of the same ribotype, namely, ribotype 3 to ET6, ribotype 6 to ET1, ribotype 7 to ET3 and ribotype 12 to ET3. Ribotyping in combination with MLEE analysis subdivided 6 of the 10 ETs.

MLEE in combination with RAPD–PCR subdivided six of the RAPD types, namely, RAPD types 1, 4, 7, 8, 9 and 11. MLEE in combination with ribotyping subdivided five of the ribotypes, namely, ribotypes 1, 4, 5, 9 and 12.

RAPD types and ribotypes correlated well with each other to a large extent. However, RAPD typing combined with ribotyping subdivided ribotypes 4, 5, 6, 9 and 12. Ribotyping subdivided RAPD types 1, 2, 4, 6 and 8.

Combining the three molecular typing techniques, the *S. aureus* isolates were subdivided into 25 distinct genotypes (Table 1). The major ET3 clone, identified in a previous study [15], could be subdivided into 6 distinct types on the basis of combinations with RAPD types and ribotypes. Of the 7 other commonest ETs accounting for 33 isolates, 5 of these could be subdivided into 2–4 combinations of RAPD types and ribotypes, and 16 combined types in all were produced from these 7 ETs. RAPD typing and ribotyping, either alone or in combination, could not discriminate between isolates in ET36 and ET39, all 8 isolates possessing an identical RAPD type and ribotype combination (Table 1). All of the isolates in ET7 appeared identical by RAPD analysis and only 1 of the 5 isolates yielded a different ribotype. Thus, it is likely that the isolates within ETs 7, 36 and 39 are of similar clonal origin, based on the evidence of the three different typing techniques.

Concordance analyses of typing techniques

The level of concordance between the different techniques was assessed by comparing all possible pairs of the 63 isolates. For MLEE and RAPD–PCR, each pair of isolates was classified by whether the isolates possessed identical or different ETs and whether they matched or mismatched in RAPD type (Table 2). In the total of 1953 possible pairwise combinations of the 63 isolates, 7% of the pairs of isolates of the same ET had the same RAPD type (match) and 74% of pairs of isolates with different ETs had different RAPD types (mismatch). The

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Table 3. *Concordance analysis of RAPD type with ribotype*

	No. of isolate pairs (proportion of total comparisons) with ribotype	
RAPD type	Match	Mismatch
Same	147 (0·08)	40 (0·02)
Different	81 (0·04)	1685 (0·86)
Concordance*	93·8 %	

* Concordance equals the sum of the same RAPD type-match and different RAPD type-mismatch entries, expressed as a percentage of the total 1953 pairwise comparisons.

overall percentage of concordant results was 80·6 % (simple matching coefficient, $S = 0{\cdot}806$) and was highly significant (G test of independence, $G = 193$, D.F. $= 1$, $P < 0{\cdot}001$). Pairwise comparison according to ET and ribotype also revealed a high level of concordance (Table 2) ($S = 80{\cdot}5$, $G = 199{\cdot}8$, D.F. $= 1$, $P < 0{\cdot}001$) and for RAPD type and ribotype there was a strikingly high level of concordance which was highly significant (Table 3) ($S = 0{\cdot}938$, $G = 552{\cdot}5$, D.F. $= 1$, $P < 0{\cdot}001$).

Diversity amongst isolates within herds

The three methods identified different molecular types amongst isolates from the same Irish herds (Table 1). In the Tipperary herd, two different combination types, namely, ET3/RAPD type 5/ribotype 7 and ET3/RAPD type 7/ribotype 4, were found and these same two molecular types were identified in the Ballyderown herd. In the Mallow herd, three different molecular types were distinguished using a combination of the three typing techniques, namely, ET3/RAPD type 4/ribotype 13, ET3/RAPD type 5/ribotype 7 and ET41/RAPD type 4/ribotype 12. These data demonstrate the presence of genetic heterogeneity among *S. aureus* isolates from a single herd.

DISCUSSION

Multilocus enzyme electrophoresis has been extensively used to index allelic diversity among human and animal pathogenic bacteria, including staphylococci [5, 15]. These studies have contributed important information to our understanding of *S. aureus* population genetics. One study utilizing MLEE, involving the analysis of over 2000 isolates from human and other hosts, demonstrated that *S. aureus*, like many other bacterial pathogens, has a predominantly clonal structure [5]. Furthermore, MLEE analysis has shown that the majority of toxic shock syndrome (TSS) cases are caused by one clone [15]. In addition, a study of over 350 isolates of worldwide origin from milk samples using MLEE showed that nearly 90 % of all isolates could be assigned to 8 ETs [16]. MLEE analysis of the *S. aureus* isolates in the present study recovered from cows in Ireland, revealed that almost all of them belonged to the common ET3 clone [16]. MLEE could distinguish 10 different ETs amongst the 63 isolates.

Recently, a large number of reports describing the use of PCR for genetic typing of medically important microorganisms have appeared [6, 8, 17]. However, in most cases, these analyses were not accompanied by detailed comparisons with the results of alternative typing procedures. Several studies of *S. aureus* have compared RAPD–PCR with only one other technique [9, 13, 17].

In the present study, MLEE, RAPD–PCR and ribotyping were utilized to study the epidemiology and genetic diversity of this important bovine pathogen. Several different primers for RAPD–PCR were tested initially. The 23-mer primer chosen was considered to be the most suitable because it resulted in a large number of amplimers over a broad size range after PCR amplification and it compared favourably in its discriminatory ability with four other primers (data not shown).

Our results reveal the presence of genetic heterogeneity among *S. aureus* strains from a single herd, with different fingerprint types occurring in different cows in the same herd (Table 1). These data agree with other studies, revealing genetic and phenotypic variability among bacterial isolates from a single herd [7, 16].

The results show that the common bovine clone of *S. aureus* identified by MLEE [16] may consist of widely distributed subclonal groups that can be detected by a combination of ribotyping and RAPD analyses. In Ireland, only six genotypic clonal types of *S. aureus* were identified amongst the isolates, using a combination of the three techniques. Worldwide, however, this study indicates that bovine mastitis may be caused by a greater number of clonal subtypes than was previously thought. The major ET type 3 identified in the USA is also the predominant type associated with *S. aureus* bovine mastitis in Ireland.

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The majority of isolates recovered from cows in Ireland had RAPD/ribotype profiles which were identified among the isolates recovered from cows in the USA. These data are consistent with the hypothesis that bovine mastitis is caused by a few specialized clones of *S. aureus* which have a broad geographic dissemination. This may have important implications in the control of mastitis caused by bovine *S. aureus*, as a rational and effective strategy for control of intramammary infections may need to be directed against clones that commonly cause disease.

Electrophoretic studies of enzymes suggest that bacterial populations consist of a limited number of independent clones [30]. In a number of previous studies, analysis of RFLPs of genomic DNA has reinforced this concept of clonality, in that, although other parts of the genome were explored, the generated results have correlated well with enzyme polymorphisms [31, 32]. These data reflect a low gene flow by genetic recombination within bacterial species. The high concordance between the MLEE data and the RAPD and ribotyping data in the present study (Tables 2, 3) suggests a low gene flow rate between bovine *S. aureus* isolates. However, the different discriminatory abilities of the three methods highlights the need for caution in inferring genetic structure from any single class of genetic markers.

Overall in this study, MLEE analysis demonstrates the sharing of clonal types of *S. aureus* between American and Irish dairy herds. The application of RAPD–PCR and ribotyping confirms these findings and identifies subclonal types within the ETs identified by MLEE, allowing for more sensitive analysis of the genetic diversity of bovine *S. aureus*. This could be of importance in the future prevention and treatment of bovine mastitis due to *S. aureus*. Further studies are in progress to determine if the *S. aureus* subtypes identified in our study differ significantly in their ability to cause disease. It is anticipated that these studies will provide valuable insight regarding the epidemiology and pathogenesis of disease caused by this important animal pathogen.

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