Multilocus Sequence Typing of Intercontinental Bovine \textit{Staphylococcus aureus} Isolates


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Received 31 December 2004/Returned for modification 13 March 2005/Accepted 17 April 2005

A total of 258 bovine-associated \textit{Staphylococcus aureus} isolates from the United States, Chile, and the United Kingdom, plus the reference isolate \textit{S. aureus} Newbould 305 (NCIMB 702892), were analyzed by multilocus sequence typing (MLST). A collection of previously characterized United Kingdom isolates were also included in the analysis. The results demonstrated that MLST is suitable for the differentiation of bovine \textit{S. aureus} isolates from various sites (milk, teat skin, milking machine unit liners, hands, and bedding) and countries. The theory of the host specificity of \textit{S. aureus} is supported by the detection of a previously undescribed clonal complex that comprised 87.4\% of the isolates studied, with representatives from all geographic locations investigated. This suggests that a single clonal group has achieved a widespread distribution and is responsible for the majority of infections. Some sequence types (STs; ST25, ST115, ST124, and ST126) demonstrated site specificity, as they were significantly \((P < 0.05)\) associated with milk or teat skin.

\textit{Staphylococcus aureus} is a major cause of bovine mastitis and is spread from cow to cow (skin or milk) via the milking machine (35, 57). Environmental spread may also occur, since strains of \textit{S. aureus} have been isolated from the environment of dairy farms and from other species that are present on dairy farms (32, 39).

A number of studies have identified potential sources of the pathogen and have investigated strain-specific differences (19, 40). The major potential sources identified were milk, body sites, and, to a lesser extent, the environment (40). Studies investigating the global population structure of bovine \textit{S. aureus} suggest that a relatively few specialized clones are responsible for the majority of intramammary infections (IMIs) (26, 55), although some authors did not report between-farm genetic homogeneity (25, 46). Most of these studies have used techniques such as phage typing (19, 40) and pulsed-field gel electrophoresis (PFGE) (7, 25) to compare isolates. These methods lack intercenter reproducibility (55). Library typing systems such as binary typing (BT) (53) and multilocus sequence typing (MLST) (31) have been developed to overcome these problems by producing results that are repeatable between laboratories and over time.

The aim of this study was to investigate the effectiveness of MLST as a method for the typing of \textit{S. aureus} isolates of bovine origin from a number of distinct geographical sources. A collection of isolates previously characterized by phage typing (19) and by PFGE and binary typing (55) was used to compare these methods to MLST. The data were then used in a preliminary analysis of the evolutionary and population biology of \textit{S. aureus} isolates of bovine origin.

MATERIALS AND METHODS

Bacterial isolates. A total of 263 coagulase-positive staphylococci were analyzed. This included 251 epidemiologically well-defined isolates from 43 herds in the United States (19) detected in milk \((n = 117)\), on teat skin \((TS; n = 75)\), in milking machine unit liners \((n = 34)\), on milkers’ hands \((n = 4)\), and in dairy cow bedding \((n = 1)\). A further 20 isolates were from milk, submitted by private veterinary practitioners to the Mastitis Laboratory at the Austral University of Chile, from 14 commonalities within four provinces of two regions in the south of the country. A further 11 isolates came from dairy cow IMIs in Somerset, United Kingdom. The reference strain \textit{S. aureus} Newbould 305 (NCIMB 702892), originally isolated from the milk of a cow with nonsevere mastitis (37) and now used for experimental challenge studies (42), was also included in the study.

Species identification. The primers developed by Stepan et al. (48), based upon a species-specific 826-bp Smal restriction fragment of genomic DNA, were used to confirm that the coagulase-positive staphylococcal isolates were \textit{S. aureus} prior to MLST.

MLST. MLST was performed as described previously (45). Briefly, the primers of Enright et al. (13) were used to amplify genes for MLST. The products were purified with a MinElute 96 UF PCR purification kit (QIAGEN) and sequenced on a Prism Genetic Analyzer 3100 DNA sequencer (Applied Biosystems, Warrington, United Kingdom) by using a BigDye Terminator (version 3.1) ready reaction cycle sequencing kit (Applied Biosystems).

Allele number and sequence types (STs) were assigned by using the \textit{S. aureus} MLST website (http://saureus.mlst.net). Trace files of putative novel alleles and the allelic profiles of novel STs were sent to the database curator for allele or ST number assignment and entry into the database.

Data analysis. The samples described above, together with a collection of previously typed isolates collected from a United Kingdom organic dairy farm (45), were included in the analyses where indicated to provide a broader perspective. The data were analyzed using chi-square tables (EpiInfo, version 6.04d; Centers for Disease Control and Prevention, Atlanta, Ga.).

Isolates were divided into clonal complexes by using the program BURST (Based Upon Related Sequence Types) (14). Further analysis of the data in...
Ancestor, ancestral sequence type; minor complex, member of a minor clonal complex. Isolates were detected is not known. Figures in the same row with different superscript symbols differ significantly (conjunction with the entire S. aureus MLST database (http://saureus.mlst.net) was done by using the enhanced version of BURST (eBURST) (16). The seven locus sequences were concatenated in the order arcC, aroE, glpF, gmk, tpi, tpi, and yqiL, to produce a 3,198-bp sequence for each ST. Phylogenetic and molecular evolutionary analyses were conducted by using MEGA (Molecular Evolutionary Genetics Analysis), version 2.1 (27). Data for STs not detected in the present study were derived from the MLST database (http://saureus.mlst.net). Splits graphs were constructed by using Splitstree, v.3.2 (12, 24).

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</table>

a All isolates from the United States were detected in cattle with subclinical mastitis, and the disease status of the cattle from which the United Kingdom and Chile isolates were detected is not known. Figures in the same row with different superscript symbols differ significantly (P < 0.05). Abbreviations: n, number of isolates; Ancestor, ancestral sequence type; minor complex, member of a minor clonal complex.

b Sequence type of reference isolate NCIMB 702892.

| TABLE 1. BURST grouping of sequence types and number detected in each country and site of isolation |

RESULTS

Two-hundred fifty-nine isolates were successfully typed: 227 from the USA (3 isolates were species other than S. aureus and 1 isolate was nontypeable), 20 from Chile, 11 from the United Kingdom, and S. aureus Newbould 305 (NCIMB 702892). This gave a typeability rate of 99.6% (50, 51).

A total of 25 different STs were detected; this included 18 novel STs isolated from the United States (n = 13), Chile (n = 4), and the United Kingdom (n = 1); the reference isolate, S. aureus Newbould 305, was ST115.

Among the American isolates, eight STs (93.0% of the isolates) were represented four or more times; among these, ST50 (n = 4) and ST349 (n = 4) were isolated only from milk, while ST115 (n = 13) was never detected in milk. There was a significant difference (P < 0.05) in the frequency of isolation of ST25, ST115, ST124, and ST126 from milk and TS (Table 1).

In 25 of the 43 herds sampled in the United States, only one ST (but of various types) was detected, with a maximum of four STs detected in any one herd. Only two sequence types (ST124 and ST25) were detected in more than four herds (16 and 27 herds, respectively; Table 1). The discriminatory power (Simpson’s index of discrimination [D]) of MLST was 0.74 (95% confidence interval [CI] = 0.61 to 0.87) for the whole collection (21, 23) (Table 2).

S. aureus was isolated from the hands of milkers of four herds; of these, three of the herds had a positive milk sample, but only one yielded the same ST as that from the milker; however, all four herds gave a positive TS sample and three yielded the same ST.

When the effects of the repeated isolation of the same ST from the same site on the same farm were taken into account, four STs were represented by four or more isolates; and of
these, only ST115 (n = 5) was never detected in milk. There remained a significant difference (P < 0.05) in the rates of isolation of ST25 and ST124 from milk and TS; however, for the other STs, too few data were available for analysis. The effects of sampling bias on the discriminatory power of MLST were not significant (Table 2), and BURST analysis of all data sets was consistent with the identification of ST97 as the ancestral ST (data not shown).

Identification of CCs. The BURST analysis (Fig. 1a) consisted of 450 isolates: the 259 isolates described above plus a further 191 isolates from an organic farm collection (45). One previously undescribed major clonal complex (CC), five minor clonal complexes, and five singleton STs were identified. A clonal complex was defined as a group of isolates which shared at least five of seven alleles with one other ST in the group; ancestral clones were assigned on the basis of the fact that they differed at a single locus from isolates with the highest number of matching genotypes. The major CC had an identifiable ancestor, whereas the minor CCs were restricted to two STs which differed at a single locus from isolates with the highest number of other genotypes (i.e., has the most SLVs). Singletons were STs which did not share five of seven alleles with any other ST.

The predicted ancestor of the major CC was ST97, and henceforth, we describe this as CC97. Assignment of the ancestral ST was supported by eBURST analysis of the data set with data from the MLST database (Fig. 1b) and was consistent with the placement of this ST at an internal node by splits decomposition analysis (Fig. 2). Bootstrap resampling of the eBURST analysis yielded a value of 100% for prediction of ST97 as the primary founder.

The newly identified CC (CC97) contained a total of 14 STs, in addition to the predicted ancestor (Fig. 1a). There were seven SLVs (ST115, ST116, ST118, ST124, ST352, ST355, and ST358), three double-locus variants (DLVs; ST127, ST347, and ST357), and four satellite STs (ST25, ST117, ST119, and ST349) which were either SLVs or DLVs of other STs in the CC. While not all STs identified as members of CC97 by BURST analysis were included in the eBURST analysis results, three further STs (ST70, ST71, and ST205) were (Fig. 1b). The discrepancy between the results of the BURST and eBURST analyses are due, in part, to the group definitions used by each algorithm and the ability to include all the data from the *S. aureus* online MLST database in the eBURST

<table>
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<th>No. of STs</th>
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<th>95% CI$^b$</th>
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<td>0.74</td>
<td>0.61–0.87</td>
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<td>227</td>
<td>19</td>
<td>0.73</td>
<td>0.70–0.76</td>
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<td>98</td>
<td>19</td>
<td>0.76</td>
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<tr>
<td>1 ST/farm</td>
<td>69</td>
<td>19</td>
<td>0.80</td>
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<td>19</td>
<td>0.80</td>
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*a* Calculated as described by Hunter and Gaston (23).

*b* Calculated as described by Grundmann et al. (21).

![FIG. 1. Analysis of bovine *S. aureus* isolates by (a) BURST and (b) eBURST analyses. In the BURST analysis green numbers denote STs from the United States, red numbers denote STs from the United Kingdom, blue numbers denote STs from Chile, and the black number is reference isolate *S. aureus* Newbould 305; figures in parentheses are the numbers of isolates. The predicted clonal ancestor is in the central ring, SLVs are in the middle (solid) ring, and DLVs are in the outer (dashed) ring; more distantly related STs are classified as satellites, and their relationships are shown as a solid (single-locus difference) or a dashed (double-locus difference) line. In the eBURST analysis, the numbers displayed are STs; the blue circle represents the primary founder, all lines represent a single-locus difference, and the size of the circles is relative to the numbers of isolates in the input data.](http://jcm.asm.org/ on October 23, 2017 by guest)
analysis. BURST analysis permits the inclusion of DLVs, i.e.,
those that share only five of seven homologous loci, whereas in
eBURST analysis, DLVs are displayed only if the intermediary
SLV is present in the data. Analysis of the data from the S.
aureus database also permitted elucidation of other potential
members of CC97 identified in other studies but not present in
our data set. Of the three additional STs identified by
eBURST, two (ST70 and ST71) were reported as bovine-as-
sociated strains isolated in The Netherlands (54).

The minor clonal complexes identified in this study (Table 1)
contained three previously detected STs; two are predicted
ancestral clones (ST30 and ST45), and one has been classified
as a singleton (ST50) (13, 15). Three singletons (ST126, ST353,
and ST9; Table 1) were represented by more than one isolate
(18, 5 and 2 isolates, respectively) and were assigned as single-
lon clones (having no clonal variants) in this collection. ST9
has been identified as the ancestral strain of a clonal complex
(14, 15), while ST126 and ST353 are previously undescribed.

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

**Phylogenetic analysis of S. aureus.** All the STs in CC97
clustered on a dendrogram of bovine-associated STs and a
diverse selection of human-associated STs (Fig. 3). The human
strains included are the ancestral STs of the 11 major methi-
cillin-resistant S. aureus clones, the ancestral STs of major
methicillin-susceptible S. aureus clones, or distantly related
STs (singletons) of these clones (10, 14). Clustering of isolates
based on the host (bovine or human) is demonstrated, as is the
putative presence of further bovine-associated CCs associated
with wide geographic spread (ST151, ST351, ST356 and ST350,
and ST354).

**Estimates of recombination.** Four SLVs had alleles that
differed at one nucleotide site from the ancestral ST, three
were unique to their ST, and all four were unique to CC97
(Table 3). Conversely, the alleles of the ancestor are present in
more than one CC, supporting the assignment of ancestral and
derived STs. Three SLVs contained alleles which differed at
one nucleotide site; of these, two were unique to their ST, and
all three were unique to CC97. However, tpi-60 (ST118) differs
from tpi-59 (ST116) by a single nucleotide polymorphism, and
it is probable this allele (tpi-60) arose as a point mutation of
tpi-59 rather than by recombination from tpi-5; this hypothesis
is supported by the splits graph of ST97 and its SLVs (Fig. 3).

**DISCUSSION**

**Typing.** The discriminatory power of MLST is similar to
those of other methods of typing S. aureus of bovine origin (1).
The typeability and Simpson’s index of discrimination (D) ob-
tained in this study were comparable to those obtained by
PFGE, phage, and binary typing in previous studies, although
inclusion of PFGE subtypes resulted in a significantly higher
index of discrimination (55). Comparison of the discriminatory
powers of MLST (D = 0.74, 95% CI = 0.61 to 0.87) and BT (D
= 0.86, 95% CI = 0.81 to 0.90) for the American isolates must
be interpreted with caution, however, as only 142 isolates were
typed by BT (55).

There was high overall agreement between MLST and
PFGE (55), as has been demonstrated with human isolates (13,
20). PFGE was, however, able to differentiate isolates of
ST124, and this could not be correlated to a herd or a site of
isolation (data not shown). This may represent evidence of the
initial stages of diversification of this strain or variations in the
 carriage of lysogenic bacteriophage. Previous studies have sug-
gested a correlation between PFGE types and clinical mani-
 festation of infections (54), but clinical data are not available
for isolates of ST124.

In contrast to binary typing, MLST and PFGE (55) results
demonstrate congruence with the site of isolation, suggesting
that MLST and PFGE are more suitable than BT for epide-
 miological investigations of bovine S. aureus isolates.

Analysis of additional and/or host-specific factors, such as
the presence of the coagulase gene (29, 49), toxin production (2, 17), and plasmid profiles (33, 36), may further improve the discriminatory power of MLST for large-scale molecular epidemiological analyses.

**Clonal diversity.** Detection of a limited number of strains per farm supports the hypothesis that *S. aureus* is a clonal organism and spreads from cow to cow (28, 57), although some studies have suggested a more environmental style of infection (25, 46). These differences may be due to the typing schemes used, but it is likely that there is also herd-specific variation due to differences in management. Effective contagious mastitis control may prevent the spread of bacteria from cow to cow, essentially leading to the generation of environmental-style pathogens and reducing the likelihood of eradication (30, 56).

The hypothesis that relatively few widely distributed clones of *S. aureus* are responsible for the majority of cases of bovine IMI (26, 54) was supported by the detection of the majority of isolates (87.4%) within one clonal complex (CC97). This may help to overcome the discrepancy of the seemingly conflicting results of between-farm genetic heterogeneity (25, 46) and the global similarity of *S. aureus* (26, 54). In this study, the dominant STs in each geographical location varied, probably due to localized selection pressures that drive the evolution of *S. aureus* in diverse hosts and environments. Yet, they all belonged to CC97, suggesting that ST97 and its derivatives are adapted not only to the mammary gland but also to the bovine environment and have achieved a worldwide distribution. Further support for a bovine-specific CC was provided by eBURST analysis, which identified two bovine-associated strains related to ST97 in the *S. aureus* MLST database. The detection of different dominant clones in various locations may have implications for the control of *S. aureus* mastitis, with the most effective strategy needing to be directed against the clones causing disease in each area (18, 52). It is possible that there are further bovine-associated (and potentially other host species-associated) CCs yet to be detected. Analysis of *S. aureus* isolates from bovine mastitis and from other hosts from a broader geographical area will help bridge this gap in knowledge.

Of the 15 members of CC97, 2 (ST97 and ST25) have been described previously (13, 15). While ST97 was not detected in United Kingdom bovine isolates in this study, it has been detected in United Kingdom ovine isolates (8) and has been classified as a singleton in United Kingdom human isolates. ST25 has been identified as the ancestral strain of a CC (10, 15) and is a DLV of a DLV of ST97. This may represent initial evidence for a pathway of strain evolution, with ST127 forming an intermediary between ancestral strains. An unrooted Bayesian tree (15) supports the closer relationship of ST25 and ST97 than to other ancestral strains.

**Recombination.** Analysis of CC97 diversification generated some unexpected findings, suggesting that there is a roughly equal chance of alleles changing by point mutation and recombination. Evidence of recombination is also provided by the interconnected “network” of STs in the splits graph of CC97 (47). This contrasts with earlier results of MLST of *S. aureus* housekeeping genes (15), which estimated that alleles are at least 15-fold more likely to change by point mutation than by recombination in the early stages of clonal diversification. These results may be due to the smaller sample size of this study compared to that of an earlier report (15), or they may indicate a slightly altered population structure of bovine *S. aureus* isolates compared with that of human *S. aureus* isolates; this warrants further investigation. The presence of alleles which are thought to have changed by recombination in more
than one strain suggests that they are being maintained in the population. It is likely that these changes (or those linked to the allelic variant detected by MLST) improve the ability of the resulting strains to colonize and infect cows (22).

**Strain adaptation.** Teat skin and milking machine unit liners were identified as important fomites for *S. aureus* IMIs based on phage typing (19). However, MLST, like PFGE, demonstrates that isolates from milk predominantly belong to different strains than isolates from teat skin, thus demonstrating not only host specificity but also site specificity (4, 55).

The STs detected on milkers’ hands were also detected in milk and on teat skin, suggesting that milkers’ hands can transmit *S. aureus* to teat skin and the mammary gland, causing IMIs. 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 specialization (8, 41). However, most authors also demonstrated that some strains of *S. aureus* were able to infect and/or colonize both humans and cows (30, 41).

The ability of the environment to act as a reservoir of *S. aureus* capable of causing IMIs was suggested in one herd, where the same ST (ST30) was detected in bedding and as a cause of IMIs. This agrees with previous reports which have demonstrated the possibility that the environment acts as a reservoir of infection (32, 40, 54). The relative importance of this, however, will be dependent on herd management. In well-managed herds, environmental transmission of *S. aureus* is more likely than contagious transmission of the organism.

**Strains for IMI models.** Analysis of the reference strain *S. aureus* Newbould 305 generated some surprising results. This strain was first detected as a cause of clinical mastitis in 1958 (37) and has been used in challenge trials ever since (5, 42) to simulate infection with a naturally occurring isolate. Infections with *S. aureus* Newbould 305 are known to be relatively gentle, with a moderate increase in somatic cell counts, virtually no clinical signs, and high cure rates after therapy (42). Interestingly, in this study *S. aureus* Newbould 305 was determined to be ST115, a strain detected on only four farms and significantly associated with teat skin. This may be indicative of the atypical nature of *S. aureus* Newbould 305 as a representative of *S. aureus* pathogenic for the udder; further work is therefore needed to fully characterize the Newbould 305 strain. Typically, intramammary *S. aureus* infections are characterized by high but variable somatic cell counts (11, 44), persistence (34, 38), variable shedding of bacteria (6, 43), and low cure rates (3, 9). While *S. aureus* Newbould 305 is convenient for use in challenge trials, it may not be representative of intramammary *S. aureus* isolates. It may be wise to catalogue the current strains of importance for use in challenge studies rather than relying on a strain that may now be better classified as a skin isolate. Potential candidate STs for challenge experiments identified in the current study are ST97, ST124, and ST126. The last two STs (ST124 and ST126) were the most common STs detected in milk, with ST97 being their predicted ancestral ST. These, plus other, perhaps more recently isolated bovine-associated strains of *S. aureus* may represent a more relevant target for the development of methods for the control of *S. aureus* IMIs. However, caution must be exercised in the use of a strain which causes more typical disease, as this is likely to lead to a more chronic infection in experimentally challenged cows. The choice of a challenge strain(s) therefore requires careful consideration.

**ACKNOWLEDGMENT**

E.M.S. was supported by a Biotechnology and Biological Sciences Research Council studentship.

**REFERENCES**


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