JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1995, p. 376–380 Vol. 33, No. 2
0095-1137/95/$04.00+0

Molecular Population Genetic Analysis of *Staphylococcus aureus* Recovered from Cows

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Received 15 July 1994/Returned for modification 9 September 1994/Accepted 15 November 1994

***Staphylococcus aureus* is one of the most common causes of bovine mastitis. To estimate genetic relationships among *S. aureus* strains recovered from cows, 357 isolates from milk samples from worldwide localities were examined for electrophoretic variation at 13 metabolic-enzyme loci. Thirty-nine electrophoretic types which represented distinctive multilocus enzyme genotypes were identified, and nearly 90% of all isolates were assigned to one of eight clones. Genetic heterogeneity was found among organisms recovered from dairy herds from which multiple isolates were obtained, indicating that the *S. aureus* population in a single herd can be multiclonal. Although humans and cows shared 7 of the 39 *S. aureus* clones, each clone was predominantly associated with one of these host species. These results are consistent with the concept of host specialization among *S. aureus* clones and imply that successful transfer of bacteria between humans and cows is limited.**

Staphylococcus aureus is one of the most frequent causes of bovine mastitis, and despite several decades of research aimed at controlling this pathogen, it remains a substantial economic problem to milk producers worldwide (25, 36, 37). Inasmuch as there is considerable genetic heterogeneity in natural populations of *S. aureus* (26–28, 35), a rational and effective strategy for control of intramammary infections may need to be directed against clones that commonly cause disease. It is therefore important to determine the genetic structure of *S. aureus* strains associated with bovine mastitis.

A variety of biochemical and molecular methods, such as bacteriophage typing, antibiotic sensitivity testing, biotyping, plasmid and chromosomal profiling with restriction endonucleases, ribotyping, and pulse-field gel electrophoresis, have been used in epidemiological investigations of human and bovine staphylococcal infections (3, 5, 16, 18, 19, 22, 23, 28, 38). These techniques are useful for classifying bacterial isolates into convenient intraspecific subsets, but most of them provide little information about estimates of overall genetic relationships among organisms.

Multilocus enzyme electrophoresis (MLEE) has been extensively used to index allelic diversity among human and animal pathogenic bacteria, including staphylococci (26–28, 35). This technique provides data from which statistical estimates of genetic diversity and overall chromosomal relationships can be obtained. These studies have led to the discovery of several important features of *S. aureus* population genetics. MLEE analysis of more than 2,000 isolates from human and other animal hosts found that *S. aureus*, like many other bacterial pathogens, has a predominantly clonal structure (28). In addition, it has been shown that the majority of cases of toxic shock syndrome (TSS) are caused by one clone (27). Recently, MLEE analysis of methicillin-resistant *S. aureus* strains documented that the chromosomally encoded *mec* gene has been widely disseminated in the staphylococcal population by horizontal transfer (26), a result confirmed by additional studies (2).

The objective of this investigation was to assess the population genetic structure of natural isolates of *S. aureus* recovered from cows. MLEE was used to determine genetic relationships among 357 *S. aureus* isolates recovered from bovine milk. Nearly 90% of these isolates were assigned to one of eight clones. The results indicated that a dairy herd may be colonized by several distinctive *S. aureus* clones. Although some *S. aureus* clones cultured from cows are closely related to isolates recovered from humans, many are not, which implies that successful transfer of this organism between humans and cows under natural conditions is limited.

MATERIALS AND METHODS

Bacterial isolates. A total of 357 isolates cultured from bovine milk samples was examined (Table 1). The 80 isolates from Pennsylvania were obtained from individual quarter milk samples from healthy cows in two unassociated dairy herds (designated PA-I and PA-II) with a high incidence of subclinical mastitis. The sample of 36 isolates from cows in Ohio was randomly drawn from a collection of 364 *S. aureus* isolates obtained during a survey of bulk milk tanks at unassociated dairy farms over 1 month (36). The remaining isolates were taken from a large collection of *S. aureus* isolates previously examined for chromosomal relationships (28). With the exception of the organisms recovered from the two Pennsylvania dairy herds, there are no known epidemiologic associations among the other isolates examined.

MLEE analysis of bacterial strains. Methods of enzyme electrophoresis have been described in detail elsewhere (26, 34). Briefly, each isolate was grown in 150 ml of tryptic soy broth (Difco Laboratories) overnight at 37°C on an orbital shaker and cells were harvested by centrifugation. The bacterial pellet was resuspended in 2 ml of lysis buffer (50 mM Tris-HCl [pH 7.5], 5 mM EDTA) which contained 100 μg of lysostaphin per ml (Sigma) and was incubated at 37°C for 45 min. The suspension was then sonicated for 30 s at 50% output (Branson model 200 sonifier; VWR Scientific) with ice bath cooling and centrifuged at 20,000 × *g* for 20 min. Enzyme-containing supernatants were either immediately resolved by electrophoresis or stored at −80°C. Following electrophoresis on starch gels, selective histochemical staining for 13 metabolic enzymes was conducted (Table 1). Distinctive mobility variants of each enzyme were numbered in order of decreasing anodal migration and equated with alleles at the corresponding chromosomal structural gene locus. Each isolate was characterized by its combination of alleles at 13 enzyme loci, and unique combinations of electromorphs were designated as electrophoretic types (ETs).

Statistical analysis. Genetic diversity and relationships among ETs were calculated with the computer programs ETDIV and ETCLUS (26). Bootstrap analysis (8) of the multilocus electrophoretic data was performed with the pro-

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TABLE 1. Properties of 357 *S. aureus* isolates from cows

Locality	No. of isolates	No. of ETs	Mean no. of alleles	$h \pm SE^a$
Argentina	11	4	1.46	0.218 ± 0.083
France	1	1	1.00	0
Louisiana	76	17	2.85	0.249 ± 0.085
New York	130	14	2.23	0.254 ± 0.079
Ohio	36	8	1.69	0.195 ± 0.072
Pennsylvania-I	41	4	1.31	0.154 ± 0.083
Pennsylvania-II	39	3	1.46	0.282 ± 0.106
Puerto Rico	4	4	1.31	0.115 ± 0.083
Tennessee	1	1	1.00	0
Texas	3	3	1.31	0.179 ± 0.097
Unknown	15	4	1.38	0.205 ± 0.083
All sources	357	39	3.77	0.259 ± 0.083

[a] Data are means ± standard errors of single locus diversity across isolates.

gram ETBOOT. Linkage disequilibrium analysis was determined with the program ETLINK. The index of association (I_A) between loci and its standard error was determined as previously described (24). The source code and compiled programs, along with instructions for use, are available by anonymous file transfer process from the Internet file server 128.118.180.78.

RESULTS

Genotypic diversity in bovine *S. aureus*. Ten of the 13 loci were polymorphic (Table 2). There was an average of 3.8 alleles per locus, and the most polymorphic loci were those which encoded esterase and shikimate dehydrogenase, with 10 and 9 alleles, respectively (Table 2). A total of 39 distinct ETs was identified, and several of these clones differed from one another at only one or two loci (Table 2). Nearly 90% of all isolates ($n = 317$) were represented by 8 clones (ETs 1, 2, 3, 5, 6, 7, 36, and 39), and 25 ETs were represented by single isolates (Table 2).

Genetic relationships among clones. The estimated relationships among multilocus genotypes are represented by the dendrogram in Fig. 1. At a genetic distance of 0.23, which corresponds to allele differences at 3 of 13 loci, the 39 ETs were assigned to six groups, designated A through F, and two single lineages, ETs 8 and 20. Groups A and B were represented by 5 and 2 ETs, respectively; group C was represented by 12 ETs; and groups D, E, and F were represented by 9, 6, and 3 ETs, respectively. Group C contained the ET (ET 3) that was most abundantly represented. The 103 isolates in ET 3 were recovered from cows in New York ($n = 68$), Pennsylvania ($n = 8$), Argentina ($n = 8$), Ohio ($n = 3$), Louisiana ($n = 3$), Texas ($n = 1$), France ($n = 1$), and unknown localities ($n = 11$). Bootstrap analysis (8) with 2,000 replications did not identify statistically significant nodes in the dendrogram.

The I_A value for all 357 isolates was 0.84 ± 0.07, indicating a high level of linkage disequilibrium among loci. When only the 39 ETs were considered in the analysis, the I_A value was reduced to 0.33 ± 0.22. These results imply that the population structure of bovine isolates of *S. aureus* fits the epidemic model described by Maynard Smith et al. (24).

Intraherd clonal variation. Heterogeneity in the *S. aureus* clones recovered from cows at two dairy farms in Pennsylvania was detected (Table 3). For example, there were four ETs identified among the 41 isolates recovered from farm PA-I. Two of these clones (ETs 36 and 39) consist of 15 isolates each, and the other two clones (ETs 3 and 5) have 8 and 3 isolates, respectively. Although the 15 *S. aureus* isolates of ET 39 were recovered only from cows at PA-I, the isolates of the other three clones (ETs 3, 5, and 36) found at this location were also recovered from non-Pennsylvania dairy farms (Table 3). Similarly, the isolates recovered from milk samples at farm PA-II were represented by three clones (ETs 1, 7, and 36), but 32 of the 39 isolates (82%) from this farm were represented by a single clone, ET 36 (Table 3). The two most common clones identified in this study (ETs 3 and 5) were not recovered from PA-II.

Comparison of bovine and human *S. aureus* clones. ETs 1, 2, 3, 4, 5, 7, and 9 in clusters A, C, D, and E have MLEE profiles identical to *S. aureus* strains from humans (26–28, 35). This result demonstrates that some clones are shared by humans and bovines. However, although clones 1, 2, 3, 5, and 7 are frequently recovered from cows, they are rarely cultured from humans (26–28, 35).

TABLE 2. Allele profiles at 10 polymorphic enzyme loci for 39 ETs of bovine *S. aureus*

ET	No. of isolates	No. of alleles at locus for enzyme[a]:									
		ACO	CAK	M1P	6PG	NSP	CAT	EST	LDH	ADH	SHK
1	24	5	5	5	5	5	5	2	5	5	5
2	8	5	5	5	5	5	5	5	5	5	5
3	103	5	5	4	5	5	5	6	5	5	3
4	2	5	5	5	5	5	5	7	5	5	3
5	64	5	5	5	5	5	5	6	5	5	3
6	7	5	5	5	6.5	5	5	3	5	5	2
7	44	5	5	5	6	5	5	3	5	5	2
8	1	5	3	8	5	5	5	5	5	5	3
9	2	5	5	4	5	5	5	6	5	5	5
10	1	6	5	4	5	5	5	5	5	5	3
11	1	5	5	7	5	5	5	8	5	5	3
12	1	5	5	5	5	5	5	3	6	5	5
13	1	5	5	7	5	5	5	8	5	5	6
14	2	5	5	5	5	5	5	3	5	5	3
15	2	5	5	5	6	5	5	3	5	5	1
16	1	5	5	5	5	5	5	3	5	5	2/3
17	1	4	5	4	5	5	5	6	5	5	3
18	1	4	5	5	5	5	5	2	5	5	0
19	1	5	5	5	5	5	5	2	5	5	3
20	1	7	3	5	5	7	5	0	4.5	5	0
21	1	5	4	5	5	5	5	10	5	5	0
22	4	5	5	5	5	5	5	3	5	5	2
23	1	5	5	5	5	5	5	2.5	5	5	0
24	1	5	5	4	5	5	5	8	5	5	3
25	1	5	7	5	5	5	5	3	5	5	7
26	1	5	5	4	5	5	7	6	5	5	3
27	1	5	5	5	5	5	5	9	5	5	5
28	1	5	9	4	5	5	5	3	5	5	5
29	1	5	5	5	5	5	5	8	5	5	3
30	1	6	5	5	5	5	5	6	5	5	3
31	1	5	7	4	5	5	5	7	5	5	5
32	1	9	5	5	6	5	5	3	5	5	2
33	3	5	5	7	5	5	5	6	5	7	3
34	1	5	5	4	5	5	5	0	5	5	0
35	1	5	7	4	5	5	5	7	5	5	3
36	52	5	7	4	5	5	5	6	5	5	5
37	1	5	5	5	5	5	6	6	5	5	5
38	1	5	5	5	5	5	5	3	5	5	5
39	15	5	7	4	5	5	5	6	5	5	9

[a] ACO, aconitase; CAK, carbamylate kinase; M1P, mannitol 1-phosphate dehydrogenase; 6PG, 6-phosphogluconate dehydrogenase; NSP, nucleoside phosphorylase; CAT, catalase; EST, α,β-naphthyl propionate esterase; LDH, L-lactate dehydrogenase; ADH, alcohol dehydrogenase; SHK, shikimic acid dehydrogenase. The enzymes glucose 6-phosphate dehydrogenase, glutamate dehydrogenase (NAD dependent), and phosphoglucose isomerase were monomorphic in all isolates examined.

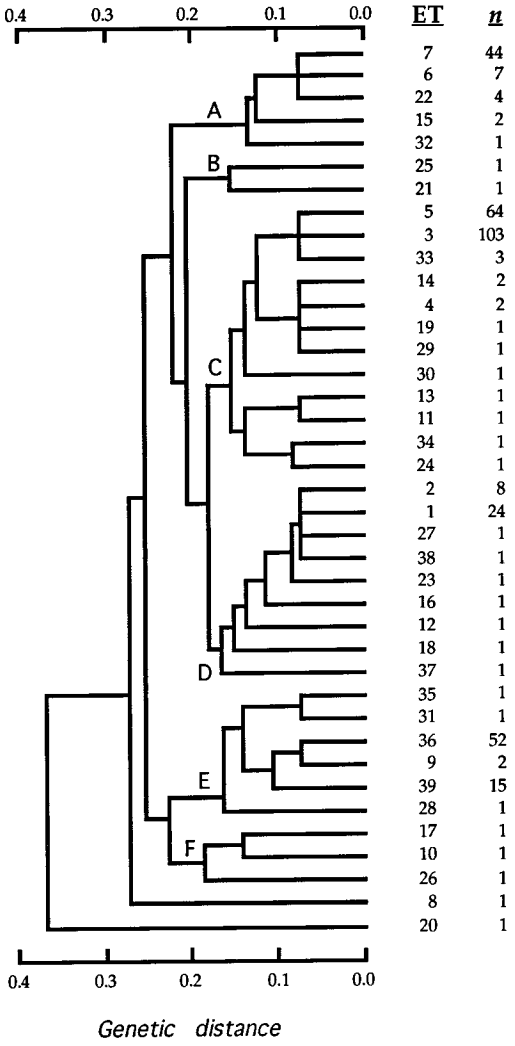

FIG. 1. Dendrogram showing estimates of genetic relationships among 39 *S. aureus* ETs on the basis of allele profiles at 13 metabolic-enzyme loci. The dendrogram was generated from a matrix of genetic distances between pairs of ETs by the average linkage method (25) with the program ETCLUS. *n*, number of isolates assigned to each ET.

DISCUSSION

Although programs directed at controlling *S. aureus* intramammary infections have been conducted for several decades, many dairy herds are infected with this pathogen. It has recently been estimated that mastitis results in a loss of $125 per cow through reduced milk production, cost of treatment, and increased culling (25). Taken together, mastitis accounts for several billion dollars of lost revenue for the dairy industry (12, 25).

Primary observations. Our results indicate that a large proportion of the *S. aureus* strains in this sample belonged to a single clone, ET 3. The most parsimonious hypothesis to account for identity in multilocus genotype is that ET 3 isolates are recently derived from a common ancestral cell which has achieved widespread distribution among cows. The overall genotypic diversity of 0.26 among the isolates from bovine milk samples is relatively low compared with those of *S. aureus* strains from humans (28), strains associated with TSS (27), or methicillin-resistant *S. aureus* strains (26). This is consistent with the results obtained in an earlier study (27) of 22 isolates from cases of animal mastitis and suggests that only a relatively small subset of extant *S. aureus* strains is able to colonize or cause clinically significant disease in cows. Moreover, since nearly 90% of the isolates examined belonged to one of eight clones, the results indicate that the great majority of bovine isolates of *S. aureus* from global sources belong to only a few clones.

TABLE 3. Frequency of isolation of common clones

Locality	No. of ETs	No. of isolates	No. of isolates of ET:								
			1	2	3	5	6	7	36	39	Other[a]
Argentina	4	11	0	0	8	0	0	0	0	0	3
France	1	1	0	0	1	0	0	0	0	0	0
Louisiana	17	76	18	4	3	37	1	0	0	0	13
New York	14	130	0	0	68	10	6	33	0	0	13
Ohio	8	36	3	4	3	14	0	5	5	0	2
Pennsylvania-I	4	41	0	0	8	3	0	0	15	15	0
Pennsylvania-II	3	39	1	0	0	0	0	6	32	0	0
Puerto Rico	4	4	1	0	0	0	0	0	0	0	3
Tennessee	1	1	0	0	0	0	0	0	0	0	1
Texas	3	3	1	0	1	0	0	0	0	0	1
Unknown	4	15	0	0	11	0	0	0	0	0	4
All sources	39	357	24	8	103	64	7	44	52	15	40

[a] Other, all clones that were represented by fewer than five isolates.

The I_A value is a measure of the degree of association between loci and has an expected value of zero for large random-mating populations of bacteria (24). In contrast, for bacterial species that are clonal at all levels because of geographic isolation or infrequent genetic recombination, the expected I_A values should be significantly different from zero. Maynard Smith et al. (24) have also described an intermediate type of population, termed an epidemic population structure. In an epidemic population, the I_A value is significantly greater than zero because of recent widespread dissemination of one or several ETs, and the statistical significance disappears when each ET is treated individually. The results show that the I_A value for bovine *S. aureus* isolates, but not ETs, is significantly greater than zero. This suggests that the population structure of bovine isolates of *S. aureus* is epidemic and that the significant levels of linkage disequilibrium among the bacterial isolates recovered from cows are due to the recent spread of only a few widely dispersed clones. These findings need to be extended by MLEE analysis of a larger sample of bovine isolates from intercontinental sources in order to minimize temporal and geographic biases.

Multiple clones within a herd. An investigation of *S. aureus* strains associated with intramammary infections revealed that isolates of one phage type were recovered from 75% of the dairy herds examined and accounted for 26% of all isolates recovered from milk (10). This led to the suggestion that several distinct phage types may be recovered from a single dairy farm (10). Similarly, a recent study of seven *S. aureus* strains from dairy cows by restriction fragment length polymorphism analysis demonstrated limited heterogeneity among isolates within a herd and suggested that the majority of cows were colonized with one restriction fragment length polymorphism type (23). These studies have been cited as evidence for the absence or low levels of intraherd bacterial variation (12, 32, 39). In contrast, other investigations have revealed genetic and phenotypic variability among bacterial isolates from one herd (10, 13, 22) and even from one cow (23). Our results clearly demonstrate the presence of genetic heterogeneity among *S. aureus* strains from a single herd (Table 3). For example, we

identified four and three clones in the PA-I and PA-II herds, respectively. The PA-II herd was predominantly colonized by clone ET 36, and most isolates from PA-I were of ETs 36 and 39 (Table 3). In addition, each herd was colonized by clones that were less abundant within the dairy herd but well represented in the population as a whole (Table 3) (Fig. 1). This result raises some important questions. For example, does temporal variation occur in the relative abundance of *S. aureus* clones found within a dairy herd? And if so, are there any differences in the relative clinical severity of mastitis?

Several of the organisms recovered from cows in the two herds (PA-I and PA-II) surveyed in depth differ from one another at one or only a few of the metabolic-enzyme loci studied for variation. Although it is a formal possibility that strains of these ETs have very recently given rise to one another, this is an unlikely scenario for two reasons. First, because MLEE does not detect all allelic variation present in metabolic-enzyme-encoding genes (4, 31, 34, 35), organisms assigned to various related ETs have undoubtedly accumulated many base pair changes in addition to those which result in allozyme variation. For example, data from analysis of nucleotide sequences of the *mdh* gene which encodes the metabolic enzyme malate dehydrogenase in *Escherichia coli* and *Salmonella* spp. have shown that allozyme analysis detected 57% of the distinctive amino acid sequences (4). On average, the generation of a new allozyme has involved 2.6 amino acid substitutions. Second, available data indicate that most of the metabolic-enzyme loci variation in bacteria and other organisms is selectively neutral or nearly so and, hence, minimally subject to convergence through adaptive evolution (6, 7, 11, 15, 30). In the absence of selection acting on the different allozymes or a hitchhiking effect (15), it is unlikely that the gene encoding a metabolic-enzyme variant would very rapidly emerge in the *S. aureus* population within a herd.

Host specificity among *S. aureus* clones. The occurrence of host and disease specificity among bacterial clones has been well described for a variety of pathogenic bacteria (35). For instance, a striking example of host specificity is found among isolates of *Bordetella bronchiseptica*, whose clones and clone families are strongly associated with either pigs or dogs (35). An extensive study of 2,077 *S. aureus* isolates from humans, cows, and sheep revealed considerable genetic diversity among strains and found that the majority of the 252 clones identified were preferentially associated with a single host species, and only 6 of 33 clonal lineages were shared between bovines and humans (28). This result suggested that the ability of the bacterium to colonize either humans or cows had evolved several times during the differentiation of *S. aureus* populations and provided strong evidence for host specificity among clones (28). On the basis of biotyping 127 *S. aureus* strains from cases of bovine mastitis in Brazil, it has recently been suggested that there is little sharing of strains between human and bovine populations (18). In contrast, other studies have implicated milkers' hands in the spread of *S. aureus* strains associated with bovine intramammary infections (10) and have shown that the organism is frequently isolated from humans in close contact with dairy cows (21). The results of this investigation show that some *S. aureus* clones may colonize both humans and bovines. However, since the clones that are frequently recovered from cows are rarely cultured from humans and vice versa, the results of this investigation are fully consistent with the general concept of host specialization among clones. We conclude that the distinctive host range of a bacterial clone is due to innate differences in the ability to successfully colonize a specific host.

There are several advantages to constructing a bacterial population genetic framework from which questions relevant to the pathobiology of *S. aureus* mastitis can be addressed. For example, whereas more than 25% of *S. aureus* isolates from bovine mammary secretions produce one or more enterotoxins or TSS toxin (14), the expression and allelic variation of these toxins among various clonal lines have not been determined. Studies of this kind are especially important since it has been established that TSS toxin-producing *S. aureus* strains from sheep that are genotypically distinct from the clone that causes the vast majority of human TSS cases (27) harbor a variant allele of the TSS toxin structural gene and that the proteins encoded by the variant alleles are functionally distinct (17). Similarly, human, ovine, and bovine isolates of *S. aureus* harbor distinct allelic variants of the structural gene for staphylococcal enterotoxin C (*sec*), and the proteins encoded by the variant *sec* alleles differ significantly in the ability to stimulate T cells (20). It is also important to determine if differences in the abilities of *S. aureus* isolates to adhere to mammalian epithelial cells (1) or bind various plasma and subepithelial matrix proteins (29) are nonrandomly distributed among the common bovine clones.

It has recently been demonstrated that cows with CA42 alleles of the bovine lymphocyte antigen have low milk somatic cell counts and are at increased risk of *S. aureus* mastitis (33). Moreover, cows with either CA42 or W7 bovine lymphocyte antigen alleles have an increased risk of infection after challenge with the Newbould 305 strain of *S. aureus* (33). These results show a close association between host genetic composition and susceptibility to *S. aureus* mastitis. It will be of considerable interest to determine if cows with CA42 or W7 bovine lymphocyte antigen alleles are equally susceptible to infection by all or only some of the common *S. aureus* clones identified in our study.

In conclusion, the results of further studies of the population genetic structure of *S. aureus* strains that cause bovine mastitis are likely to provide additional important insights regarding disease pathogenesis and epidemiology and may also be of considerable relevance to the development of immunoprophylactic agents against bovine mastitis (9, 40).

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