Distribution of Streptococcal Inhibitor of Complement Variants in Pharyngitis and Invasive Isolates in an Epidemic of Serotype M1 Group A *Streptococcus* Infection

Nancy P. Hoe,[1] Jaana Vuopio-Varkila,[2] Martti Vaara,[2,3] Diana Grigsby,[4,a] David De Lorenzo,[5] Yun-Xin Fu,[5] Shu-Jun Dou,[4] Xi Pan,[4] Kazumitsu Nakashima,[4,a] and James M. Musser[1]

[1]Laboratory of Human Bacterial Pathogenesis, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana; [2]Department of Bacteriology, National Public Health Institute, and [3]Section of Clinical Microbiology, Helsinki University Central Hospital Laboratory Diagnostics, Helsinki, Finland; [4]Department of Pathology, Baylor College of Medicine, and [5]Human Genetics Center, The University of Texas Health Sciences Center, Houston

Streptococcal inhibitor of complement (Sic) is a highly polymorphic extracellular protein made predominantly by serotype M1 group A *Streptococcus* (GAS). New variants of the Sic protein frequently appear in M1 epidemics as a result of positive natural selection. To gain further understanding of the molecular basis of M1 epidemics, the *sic* gene was sequenced from 471 pharyngitis and 127 pyogenic and blood isolates recovered from 598 patients living in metropolitan Helsinki, Finland, during a 37-month population-based surveillance study. Most M1 GAS subclones recovered from pyogenic infections and blood were abundantly represented in the pool of subclones causing pharyngitis. Alleles shared among the pharyngitis, pyogenic, and blood samples were identified in throat isolates a mean of 9.8 months before their recovery from pyogenic infections and blood, which indicates that selection of most *sic* variants occurs on mucosal surfaces. In contrast, no variation was identified in the *emm* and *covR/covS* genes.

Although insight has been obtained about the microbial and host factors that contribute to epidemic waves of some pathogens, for most organisms molecular explanations for changes in disease frequency and severity are poorly understood. Molecular explanation of these phenomena is critical to the development of rational methods to limit pathogen emergence, resurgence, and dissemination.

Group A *Streptococcus* (GAS) is a gram-positive human pathogen that causes pharyngitis ("strep throat"), invasive infections such as sepsis and necrotizing fasciitis, and postinfection sequelae that include rheumatic heart disease and glomerulonephritis. GAS is genetically highly heterogeneous. For example, >100 distinct serotypes have been identified on the basis of antigenic differences in the M protein, an antiphagocytic surface molecule that is an important virulence factor [1]. However, it has been recognized for many decades that GAS serotypes expressing relatively few M types cause the majority of human invasive infections. In particular, serotype M1 organisms are responsible for an inordinately large proportion of invasive infections [2].

The results of several recent studies have suggested that the streptococcal inhibitor of complement (Sic), an extracellular protein made by M1 GAS, contributes to the abundance of these isolates in invasive episodes and participates in epidemics [3, 4]. Sic inhibits the membrane attack complex of human complement in vitro [5], but the in vivo function of this protein is unknown. The *sic* gene is present in all serotype M1 GAS isolates, whereas most strains of other M protein types lack this gene [5]. Importantly, contrary to conventional wisdom, epidemics of invasive M1 infections involve a remarkably heterogeneous array of subclones defined by Sic structural variants that increase in frequency very rapidly [4]. Approximately 50% of humans have serum antibody and ~90% have mucosal antibody that reacts specifically with polymorphic regions of Sic [6]. Mouse model studies conducted with a Sic-negative isogenic mutant GAS strain have revealed that Sic contributes to long-term persistence in the upper respiratory tract [7].

Despite these advances, many aspects of the contribution of Sic to the epidemic behavior and pathogenesis of M1 GAS remain unknown. For example, the relationship between Sic variants and other GAS virulence factors in M1 strains recov-

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[a] Present affiliations: Boston University School of Public Health, Boston, Massachusetts (D.G.); Department of Respiratory Diseases, Chubu National Hospital, Aichi, Japan (K.N.).

Reprints or correspondence: Dr. James M. Musser, Laboratory of Human Bacterial Pathogenesis, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 903 S. 4th St., Hamilton, MT 59840 (jmusser@niaid.nih.gov).

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ered from host mucosal surfaces and pyogenic and invasive episodes has not been delineated in a population-based longitudinal study. The recovery of M1 GAS isolates from invasive and noninvasive infections during a recent epidemic of disease in Finland provided an opportunity to address these issues.

Materials and Methods

Bacterial strains. The 471 serotype M1 GAS isolates causing pharyngitis and the 127 M1 GAS isolates obtained from patients with pyogenic and bloodstream infections were collected in metropolitan Helsinki, Finland (population 1.3 million), as part of a nationwide population-based surveillance study of GAS disease. The GAS isolates were recovered by the Helsinki University Clinical Microbiology Laboratory, Aurora Hospital Microbiology Laboratory, and Jorvi Hospital Microbiology Laboratory, Espoo (a community located in the Helsinki metropolitan area). Together, these laboratories are responsible for virtually all throat culture diagnoses of GAS pharyngitis in metropolitan Helsinki. Approximately 70% of GAS pharyngitis episodes in this area are diagnosed by culture rather than by direct antigen detection or other methods. From June 1994 through June 1997, all pyogenic and blood isolates and the first 20–100 consecutive pharyngitis isolates cultured every month from each laboratory were transported to the National Public Health Institute in Helsinki for serotyping and archiving. The 471 M1 GAS pharyngitis isolates included 125 organisms analyzed earlier [4].

GAS gene characterization. Polymerase chain reaction (PCR) amplification of the *sic* and *emm1* genes was conducted as described elsewhere [3, 4, 8, 9]. The *covR* and *covS* genes were amplified by PCR from M1 GAS genomic DNA, using the following oligonucleotide primers: *covR*1 (forward), 5′-CCAGCAGGTCAAAATTGGG-3′; *covR*2 (reverse), 5′-TAGATAGTCGTTTTGGTAACG-3′; *covS*1 (forward), 5′-TATATCCAAACAGTGCGTGG-3′; and *covS*2 (reverse), 5′-GATTACATACTATACCTGTCAC-3′. PCR amplification of the *covR* and *covS* genes was performed with a GeneAmp PCR System 9700 thermal cycler (Perkin-Elmer). The thermal cycler parameters used were 30 cycles of annealing at 55°C for 1 min, 3 s and extension at 72°C for 1 min, 15 s for *covR* and 30 cycles of annealing at 45°C for 1 min, 15 s and extension at 72°C for 3 min, 45 s for *covS*. Each PCR reaction was preceded by a denaturation step at 94°C for 5 min and was terminated by a primer extension step at 72°C for 7 min. Each cycle had an initial denaturation step of 94°C for 1 min. Unincorporated nucleotides and primers were separated from amplified DNA with a QIAquick 96 PCR purification kit (QIAGEN). Sequencing reactions were performed with the BigDye terminator cycle sequencing ready reaction kit (PE Applied Biosystems), using the following oligonucleotide primers: *covR*3, 5′-ACGACTATATTGTTAAACCG-3′; *covS*3, 5′-ATTTCGTCAGGAGATGAAATC-3′; *covS*4, 5′-GTCTATAACATTGATAAACAGATG-3′; and *covS*5, 5′-AGCAGGCTTTGATGATTCTG-3′. The unincorporated dye terminators and primers were separated from the extension products by spin column purification in a 96-well format (Centri-Sep). All genes were sequenced with a Prism 3700 capillary DNA analyzer (PE Applied Biosystems). The sequence data were assembled and edited with Sequencher software, version 3.1.1 (Gene Codes).

Phylogenetic analysis of sic. Phylogenetic trees were reconstructed with PAUP* version 4.0 (Sinauer Associates) [10]. The unrooted cladograms for the *sic* nucleotide sequences were obtained by the maximum parsimony method [11–13]. The trees obtained are unrooted because the ancestral sequence of the samples is not known. Insertions and deletions were weighted equally with nucleotide substitutions.

Results

sic *alleles in pharyngitis, pyogenic, and blood isolates.* There are currently 2 general models of the genetic relationship of invasive and noninvasive bacterial strains recovered at the same time in the same geographic area. Limited data [14–17] suggest that the frequency of occurrence of a GAS clone among invasive disease isolates is related to its abundance among pharyngitis isolates in the same geographic region. In contrast, studies with *Neisseria meningitidis* have shown that the common invasive clone is rarely identified in carriers [18–20]. However, the previous GAS studies were conducted before the discovery of variation in Sic.

The extensive variation in the *sic* gene and Sic protein among serotype M1 GAS and the demonstration that the protein contributes to persistence of infection in the upper respiratory tract of mice suggested that there is not a simple relationship between Sic variants represented among pharyngitis and invasive isolates circulating in the same community. To study this relationship, we sequenced the *sic* gene in 471 M1 GAS pharyngitis isolates and in all available pyogenic and blood isolates (n = 127) recovered during a 37-month surveillance period in metropolitan Helsinki, a time when this region was experiencing an epidemic of GAS infections caused by M1 strains (figure 1) [17]. The pharyngitis organisms are a random sample of all M1 GAS pharyngitis strains cultured in this period but represent <25% of all cases caused by M1 strains. Fifty-four *sic* alleles

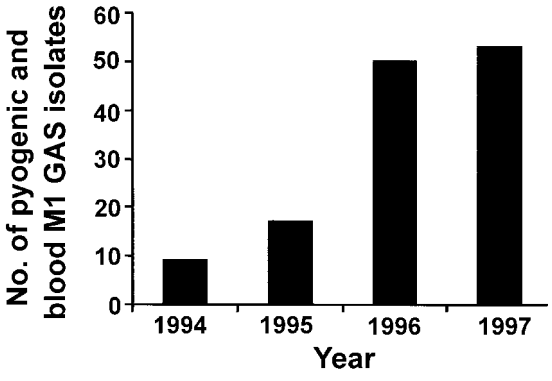

Figure 1. Blood and pyogenic serotype M1 group A *Streptococcus* (GAS) isolates recovered in metropolitan Helsinki, Finland, from June 1994 through June 1997. The numbers of isolates identified in 1994 and 1997 represent those recovered from June through December and January through June, respectively.

Table 1. Time elapsed between initial identification of streptococcal inhibitor of complement *(sic)* alleles in pharyngitis and invasive M1 group A *Streptococcus* isolates in metropolitan Helsinki, Finland.

sic allele	No. (%) of M1 isolates with each allele		Time elapsed, months[b]
	Pharyngitis	Invasive[a]	
1.01	213 (45.2)	60 (47.2)	6
1.102	35 (7.4)	13 (10.2)	12
1.13	29 (6.2)	5 (3.9)	5
1.02	21 (4.5)	7 (5.5)	3
1.107	16 (3.4)	5 (3.9)	19
1.59	15 (3.2)	4 (3.2)	15
1.73	13 (2.8)	5 (3.9)	0
1.133	13 (2.8)	2 (1.6)	8
Other[c]	50 (10.6)	20 (15.7)	0–30

[a] Includes pyogenic and blood isolates.
[b] Mean time elapsed was 9.8 months.
[c] Includes 12 uncommon *sic* alleles, each representing <2% of pharyngitis isolates.

were identified in the 471 isolates, including 14 alleles that were not represented in our database of 300 *sic* alleles. Twenty of the 26 *sic* alleles identified in the 127 pyogenic and blood isolates were also represented among the 54 *sic* alleles in the pharyngitis sample. Hence, 6 *sic* alleles were found only in the pyogenic and blood isolates, and 24 *sic* alleles were identified only in the pharyngitis isolates. The proportions of isolates represented by the 4 more abundant shared alleles *(sic1.01, sic1.102, sic1.13,* and *sic1.02)* were almost identical in the pharyngitis and pyogenic and blood isolates, being 62% and 60%, respectively.

Analysis of the temporal distribution of the 20 alleles shared between pharyngitis and pyogenic and blood isolates revealed that 15 of them were identified in throat culture specimens a mean of 9.8 months before their representation among pyogenic and blood isolates (table 1). These results show that most abundant M1 GAS subclones causing pyogenic infections and septicemia are common in the pool of subclones causing pharyngitis. In addition, the results indicate that selection of most Sic variants occurs on human mucosal surfaces.

emm1 *sequences in pharyngitis and invasive isolates.* It was reported in an earlier study [4] that structural changes in the antiphagocytic M1 protein virulence factor do not contribute to alterations in the frequency of pharyngitis or invasive disease. This conclusion was based on sequence analysis of the region of the *emm1* gene encoding the hypervariable aminoterminus in 120 of the 127 pyogenic and blood isolates. All isolates had the *emm1.0* allele [4]. In addition, the *emm1* gene had been sequenced in 117 of 125 pharyngitis isolates analyzed earlier, and only 3 alleles were identified *(emm1.0, emm1.5,* and *emm1.23)*. Virtually all (98%) of the 117 isolates had *emm1.0*. However, it was possible that certain *emm1* alleles had increased in frequency in the larger sample of pharyngitis strains and that these variants had been undetected, because only 117 of the 471 pharyngitis isolates were analyzed. To test this hypothesis, and to delineate more fully the relationships between *emm1* sequences in pharyngitis and pyogenic and blood isolates, the variable region of the *emm1* gene was sequenced in 330 of the additional 346 pharyngitis isolates collected in the 37-month study. Virtually all strains (325 [98%] of 330) had allele *emm1.0*. Alleles *emm1.20, emm1.34, emm1.35, emm1.36,* and *emm1.37* were identified in 1 strain each. Compared with the *emm1.0* allele encoding variant M1.0 [21], each of these alleles is characterized by single nucleotide changes resulting in single amino acid substitutions, with the exception of *emm1.37,* which is characterized by a 3–amino acid insertion in M protein (figure 2). Thus, although new M1 variants arose sporadically in the pharyngeal isolates, they never increased significantly in frequency and were not detected in the pyogenic and blood isolates recovered during the same time period.

Amino acid replacements have been detected in M1 protein regions other than the aminoterminus [8, 21, 22], and some of these structural changes correlate with altered opsonophagocytosis [23]. This region of the *emm1* gene is characterized by several sequence repeats, designated A, C, and D (figure 2) [21]. To address the possibility that structural variation in this region of the M1 protein contributed to changes in strain abundance, we sequenced nucleotides 394–1188 of the *emm1* gene in 123 of the 471 pharyngitis strains and in all 127 pyogenic and blood isolates. Five variants were identified (figure 2). One invasive and 1 pharyngitis isolate were characterized by an A→C change at nucleotide 954, which resulted in a Glu918→Asp918 replacement at amino acid residue 318. Two pharyngitis isolates were characterized by a 252-bp deletion, resulting in the deletion of 84 amino acids. The deletion spanned nucleotides 724–975, deleting amino acids 242–325, in 1 isolate and spanned nucleotides 784–1036, deleting amino acids 262–345, in the other isolate. Both isolates had an additional 72-bp deletion spanning nucleotides 451–522, resulting in the deletion of 24 amino acids (151–174). Finally, 1 pharyngitis isolate was characterized by the insertion of an additional 42–amino acid C2 repeat (figure 2). None of the 5 isolates had alterations in the aminoterminal region. Taken together, our data indicate that, although several M1 variants were identified, none increased appreciably in frequency in the human population.

Lack of variation in the covR *and* covS *genes in pharyngitis and pyogenic and blood isolates.* The *covR*/*covS* locus encodes a newly described 2-component regulatory gene system that represses the expression of several proven or putative virulence factors, including streptokinase, streptolysin S, streptococcal pyrogenic exotoxin B (extracellular cysteine protease), and hyaluronic acid capsule [24–27]. The *covR* and *covS* genes are contiguous in the M1 GAS chromosome. The increased expression of these factors by an isogenic *covR* mutant strain has been reported to enhance virulence in mouse models of skin and systemic infection [24, 26]. Recently, it was reported that M1 GAS strains recovered from the blood and spleen of mice infected subcutaneously with a wild-type strain had truncation mutations in *covR* and *covS* [28]. These data suggest that allelic variation in *covR* or *covS* enhances the ability of a mucosal strain to persist or to invade deeper sites in humans. To de-

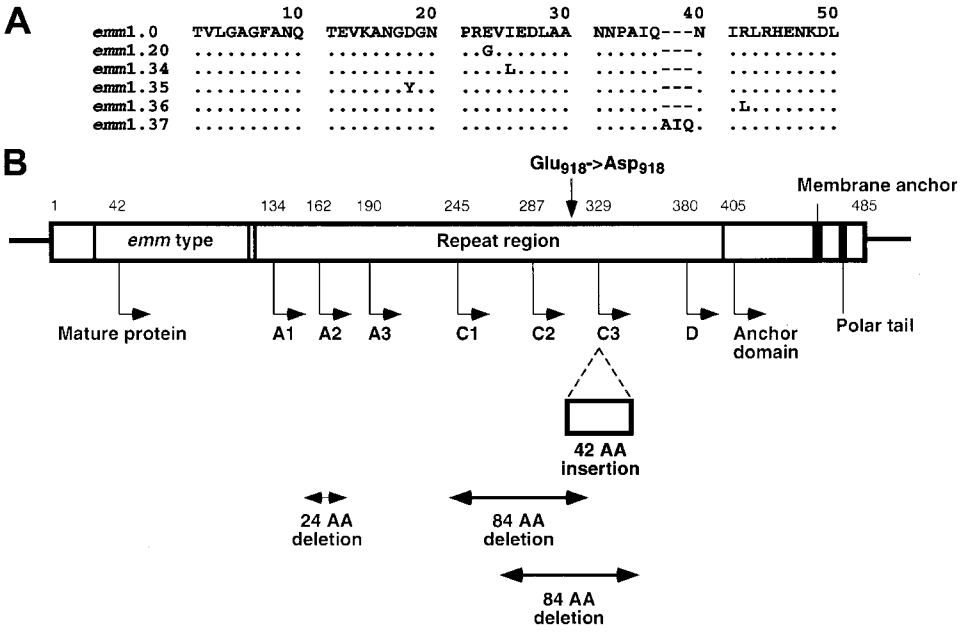

Figure 2. Variation in the *emm1* gene identified in pharyngitis and pyogenic and blood group A *Streptococcus* (GAS) isolates recovered in metropolitan Helsinki, Finland. *A,* Alignment of inferred N-terminal amino acid ("AA") sequences of 5 alleles of *emm1.0* identified in pharyngitis GAS isolates in metropolitan Helsinki. The region shown represents amino acids 27–73 (Genbank accession number X07933). Amino acid residues identical to those encoded by *emm1.0* are represented by periods. *B,* Schematic representation of the polymorphisms identified in the nonaminoterminus region of the *emm1.0* gene. *Double-headed arrows,* deletions spanning amino acids 151–174, 242–325, and 262–345; *box,* insertion of an additional C2 repeat (42 amino acids); *A1–A3, C1–C3,* and *D,* repeat sequences A, C, and D, respectively. Protein features and domain designations are taken from [21].

termine whether specific *covR* or *covS* alleles occurred preferentially among pharyngitis or pyogenic and blood isolates or increased in frequency over time in either of these populations, we sequenced the entire coding region of *covR* and *covS* and 360 bp upstream of the *covR* start codon in 123 pharyngitis and 127 pyogenic and blood isolates. All 250 isolates differed from the M1 *covS* genome sequence by an A→G change at nucleotide 994 relative to the *covS* start site, which resulted in the replacement of valine for isoleucine at amino acid residue 332. The sequence of the *covR* gene was otherwise identical to the M1 genome sequence in 99% (122 of 123) of the pharyngitis isolates and 98% (125 of 127) of the other isolates. The variant pharyngitis isolate and the 2 variant invasive isolates were each characterized by a single nucleotide change that resulted in an amino acid replacement: Gly61→Ser61, Arg158→Cys158, and Gly272→Val272, respectively. No other polymorphisms were identified in the *covS* gene. Hence, allelic variation in *covR* or *covS* was exceedingly rare, and variants in these genes were not preferentially associated with pharyngitis or pyogenic and septicemia episodes or linked to an increased frequency of recovery from human infections over time.

Phylogenetic analysis of sic. The availability of the *sic* allele data for these population-based samples of pharyngitis and pyogenic and blood GAS isolates permitted us to examine the relationships between alleles recovered from the distinct clinical conditions. Specifically, we tested the hypothesis that Sic variants recovered from pyogenic and blood infections are a nonrandom subset of pharyngitis-associated variants. If differences in the *sic* gene are responsible for the invasive or noninvasive capacity of GAS strains, analysis of the phylogenetic trees should reveal spatial differentiation between *sic* alleles in strains from the disease categories. That is, *sic* alleles in invasive isolates would cluster together. A phylogenetic tree was constructed on the basis of the nucleotide changes and the insertion and deletion differences identified in all *sic* alleles in the sample period (figure 3). Five of the 6 invasive-specific alleles *(sic1.04, sic1.113, sic1.116, sic1.134,* and *sic1.147)* were located in several branches of the tree and did not cluster together as a distinct population. One invasive-specific allele *(sic1.158),* characterized by the insertion of a "T" at nucleotide 121 of the Sic coding region (numbering as in [5]), is predicted to produce a truncated Sic product of 8 amino acids and is not included in the phylogenetic analysis. Moreover, there are clusters of alleles that contain both invasive-specific alleles and pharyngitis-specific alleles (for example, *sic1.113* and *sic1.267,* and *sic1.116* and *sic1.162*). These results mean that, on average, *sic* alleles in pyogenic and blood strains are more closely related to pharyngitis-associated *sic* alleles than they are to one another. Identical results were obtained when trees were constructed with nucleotide polymorphisms or insertions and deletions consid-

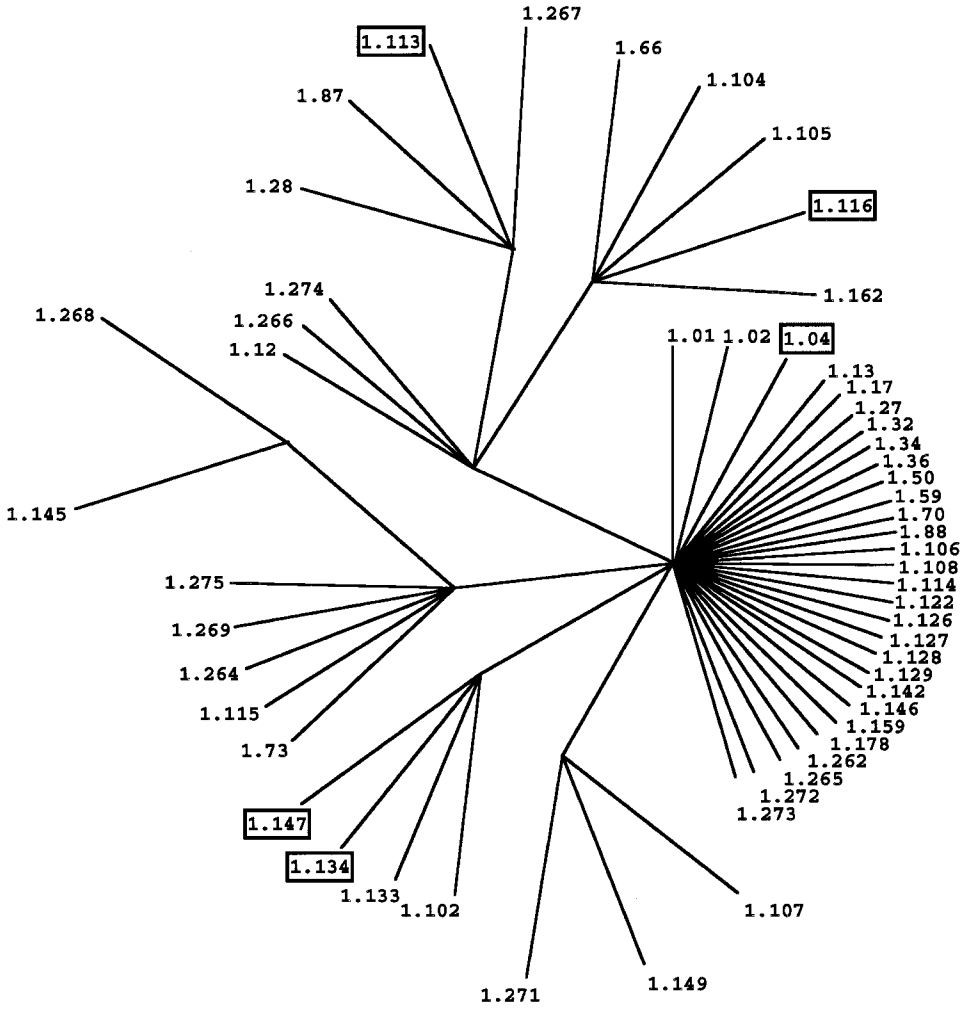

Figure 3. Evolutionary tree for streptococcal inhibitor of complement *(sic)* alleles. The unrooted parsimony tree was generated from all *sic* alleles identified by the analysis of pharyngitis and pyogenic and blood group A *Streptococcus* isolates recovered in the 37-month study period in metropolitan Helsinki, Finland. *Boxed alleles,* variants specific to pyogenic and blood isolates.

ered separately, or when distinct segments of the molecule were analyzed separately (data not shown). These results effectively refute the hypothesis.

Discussion

Insights into host and pathogen molecular factors contributing to epidemics of human infectious diseases are limited. Our analysis provides new information about the molecular population genetics of serotype M1 organisms causing pharyngitis and invasive disease episodes in an epidemic. First, Sic variants that are common among invasive isolates are also abundant in pharyngitis isolates in the same geographic area. This finding contrasts with the situation for *N. meningitidis,* in which clones or subclones responsible for invasive episodes are relatively rarely found in abundance on the mucosa of human hosts in the same geographic area [20]. Second, although common Sic variants were abundant in both the pharyngitis and invasive samples, a substantially larger number of Sic variants is associated with host mucosal infection than with invasive episodes. Inasmuch as the pharyngitis samples represented <25% of the total pharyngitis cases caused by M1 isolates in the study population, our data indicate that the number of distinct Sic variants extant on the mucosa in the human population experiencing an M1 epidemic wave is exceedingly large. These observations suggest enhanced constraint on Sic variants associated with invasive infections, although more studies will be required to evaluate this idea. Third, new Sic variants occurred among the pharyngitis isolates many months before their detection in GAS strains causing invasive disease. The molecular mechanisms responsible for this observation are not known, but it is reasonable to speculate that mucosal dissem-

ination of a subclone marked by a new Sic variant is required in the human population before a host susceptible to an invasive episode is encountered, presumably by chance. Sic may enhance the ability of serotype M1 GAS to survive on the mucosa and may partly explain the predominance of serotype M1 GAS in invasive infections, relative to other serotypes associated with invasive disease that lack Sic (e.g., serotype M3) [2]. An alternative, but not mutually exclusive, hypothesis is that molecular changes accumulate in the organism during person-to-person passage on the mucosal surface, resulting in selection of a bacterium with enhanced host invasion capacity. We currently lack sufficient data to rule out either hypothesis, although we note that blood passage of GAS is well known to select for organisms with enhanced virulence. Regardless, the observation that many months elapse before Sic-marked subclones responsible for abundant pharyngitis episodes are recovered from invasive infections in the same human population may well have practical public health ramifications.

The *covR*/*covS* locus encodes a 2-component regulatory system that represses the expression of hyaluronic acid capsule, streptolysin S, streptokinase, mitogenic factor, and streptococcal pyrogenic exotoxin B [24–27, 29]. Genetic inactivation of this system results in increased expression of these proven and putative virulence factors and enhances virulence in mouse models of skin and systemic infection [24–27, 29]. Engleberg et al. [28] have reported that *covR* and *covS* truncation mutations are present in M1 GAS strains cultured from the blood and spleen of mice infected subcutaneously. Inactivation of *covR* has also been reported to reduce GAS internalization into Hep-2 epithelial cells grown in culture [30]. It has been speculated that invasive disease episodes may be facilitated by mutations in the genes encoding *covR* or *covS* [28]. Therefore, we anticipated the identification of large numbers of *covR*/*covS* mutations among the organisms recovered from humans with pharyngitis or invasive infections. However, this was not the case. Hence, if up-regulation of these factors is commonly operative in the course of human infections, another molecular mechanism is responsible.

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