

## Molecular Subtyping and Characterization of Bovine and Human *Streptococcus agalactiae* Isolates

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***Streptococcus agalactiae* causes severe invasive disease in humans and mastitis in cattle. Temporally matched bovine milk isolates and clinical human invasive isolates (52 each) collected in New York State over 18 months were characterized by molecular subtyping and phenotypic methods to probe the interspecies transmission potential of this species. EcoRI ribotyping differentiated 17 ribotypes, and DNA sequencing of the housekeeping gene *sodA* and the putative virulence gene *hylB* differentiated 7 and 17 allelic types, respectively. Human and bovine isolates were not randomly distributed between ribotypes or *hylB* and *sodA* clusters. The combined analysis of all subtyping data allowed the differentiation of 39 clonal groups; 26 groups contained only bovine isolates, and 2 groups contained both human and bovine isolates. The EcoRI ribotype diversity among bovine isolates (Simpson's numerical index of discrimination [mean  $\pm$  standard deviation],  $0.90 \pm 0.05$ ) being significantly higher than that among human isolates ( $0.42 \pm 0.15$ ) further supports that these isolates represent distinct populations. Eight human isolates, but no bovine isolates, showed an IS1548 transposon insertion in *hylB*, which encodes a hyaluronidase. Based on data for 43 representative isolates, human isolates, on average, showed lower hyaluronidase activities than bovine isolates. Isolates with the IS1548 insertion in *hylB* showed no hyaluronidase activity. Human and bovine isolates did not differ in their abilities to invade HeLa human epithelial cells. Our data show that (i) EcoRI ribotyping, combined with *hylB* and *sodA* sequencing, provides a discriminatory subtype analysis of *S. agalactiae*; (ii) most human invasive and bovine *S. agalactiae* isolates represent distinct subtypes, suggesting limited interspecies transmission; and (iii) hyaluronidase activity is not required for all human infections.**

*Streptococcus agalactiae* (Lancefield group B) causes disease in both humans and cattle. For humans, *S. agalactiae* is responsible for severe invasive disease in adults and neonates, as well as asymptomatic infections in women (2). *S. agalactiae* has been recognized as the leading cause of neonatal septicemia and meningitis; it has been estimated to have caused 16,880 human cases, including 1,650 deaths, in the United States alone in 1998 (41). The colonization of humans can occur through a variety of mechanisms, including maternal vaginal carriage and breast-feeding (2). In cattle, *S. agalactiae* causes clinical and subclinical infections of the mammary gland (mastitis) (27). Initial studies have suggested that interspecies transmission between cattle and humans is possible. For example, experimental inoculation studies have shown that human *S. agalactiae* isolates can infect and cause clinical mastitis in cattle (23, 46). Tissue culture studies have also shown that both bovine and human *S. agalactiae* isolates can adhere to bovine epithelial cells (5). These data did not provide any information on epidemiological mechanisms or the likelihood of interspecies transmission of *S. agalactiae*, nor do they provide any conclusive evidence as to the ability of bovine-associated *S. agalactiae* to cause human invasive infections.

*S. agalactiae* isolates of human origin have been character-

ized by using a variety of methods, including biochemical characterization, serotyping, multilocus enzyme electrophoresis, randomly amplified polymorphic DNA (RAPD) analysis, ribotyping, and, more recently, multilocus sequence typing (MLST) (4, 8, 18, 20, 25, 39). Based on multilocus enzyme electrophoresis data, Helmig et al. (19) were able to divide 118 human *S. agalactiae* isolates from Denmark into two major clusters. One cluster contained isolates that were almost all from patients with clinical symptoms, while the other cluster contained isolates mostly from asymptomatic carriers. Comparative subtyping studies using both classical phenotypic (e.g., serotyping) and DNA sequence-based methods to examine human and bovine *S. agalactiae* isolates have provided only limited insight into the likelihood of cattle serving as a reservoir or direct source of *S. agalactiae* strains in human invasive infections (4, 24, 32). For example, serotyping and ribotyping provided some evidence that cattle and asymptomatic human carriers that are present in the same locality can carry indistinguishable *S. agalactiae* subtypes (24). RAPD typing showed that 223 *S. agalactiae* isolates from asymptomatic human carriers and from bovines generally represented different subtypes; however, this study also found one human isolate and one bovine isolate that shared an identical RAPD type, even though these isolates had different serotypes (32). A recent MLST study also showed that animal and human invasive and carriage-associated isolates represent largely separate populations, even though a specific human hyperinvasive clone ap-

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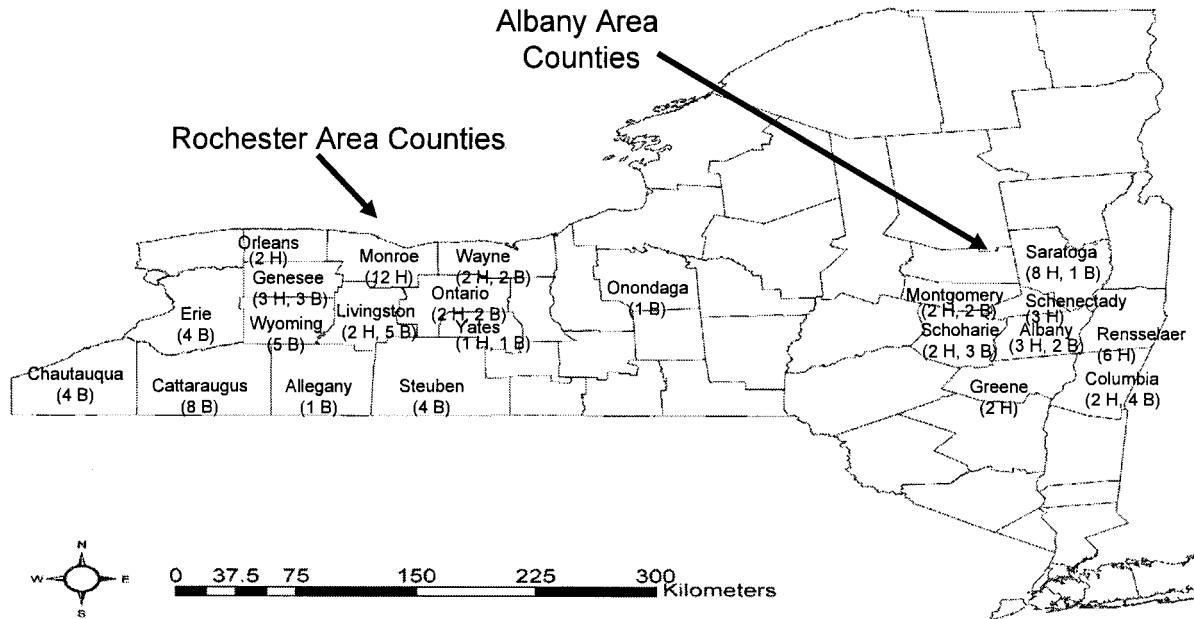


FIG. 1. Geographic distribution, across New York State, of the 104 *S. agalactiae* isolates included in this study. The numbers of human (H) and bovine isolates (B) are indicated in parentheses below the county names.

pears to have emerged from a bovine ancestor (4). Similarly, a subtyping study using phenotypic methods showed that human and bovine *S. agalactiae* strains isolated in Kenya appear to represent largely separate populations (34). Most of these previous studies have focused on (24, 32, 34) or included (4) human carriage isolates, thus providing limited insight into the likelihood of cattle serving as a source of human invasive disease-associated *S. agalactiae*. Thus, the goal of this study was to characterize temporally and geographically matched *S. agalactiae* isolates from human invasive and bovine udder infections by using standardized and reproducible molecular subtyping methods. While the use of *S. agalactiae* isolate sets that include human invasive and animal isolates collected in different regions, countries, or continents and/or during different times can provide important insights into the evolution of this pathogen (4), the characterization of temporally and geographically matched isolates is critical to assess the likelihood and potential for interspecies transmission of *S. agalactiae*.

#### MATERIALS AND METHODS

***S. agalactiae* isolates.** A total of 194 human clinical isolates of *S. agalactiae* were obtained through the U.S. Centers for Disease Control and Prevention Emerging Infections Program (EIP) conducted by the New York State Department of Health. As part of this active surveillance program, group B *Streptococcus* isolates from invasive human infections were collected from different counties in two distinct geographic regions of New York State (Rochester and Albany). A total of 109 isolates were obtained from counties participating in the Rochester area surveillance program, including Genesee, Livingston, Monroe, Ontario, Orleans, Wayne, and Yates counties. The other 85 isolates were obtained from counties participating in the Albany area surveillance program, including Albany, Columbia, Greene, Montgomery, Rensselaer, Saratoga, Schenectady, and Schoharie counties (Fig. 1). A total of 236 bovine *S. agalactiae* isolates, representing 52 farms, were obtained from the Cornell University Quality Milk Production Services mastitis control program. As part of this program, *S. agalactiae* strains were isolated from cows as previously described by Wilson et al. (48). The *S. agalactiae* strains isolated for this study represented the predominant organism found in a given quarter milk sample and are thus the likely

causative agent of clinical or subclinical mastitis in these animals. Geographically, these isolates were gathered from the aforementioned EIP surveillance counties, as well as from geographically close non-EIP counties (i.e., Allegany, Cattaraugus, Chautauqua, Erie, Onondaga, Steuben, and Wyoming counties) (Fig. 1). A total of 52 bovine isolates, 1 per farm, were chosen from the larger pool of 236 isolates for this study. An equal number of human isolates were temporally matched with these 52 bovine isolates and selected as representative of the geographic diversity for the entire pool of human isolates. Thus, a total of 104 isolates were used for this study (Fig. 1). All isolates were stored at  $-80^{\circ}\text{C}$  in brain heart infusion (BHI) broth (Becton Dickinson, Sparks, Md.) with 15% glycerol.

**Automated ribotyping.** Automated EcoRI ribotyping was performed using the RiboPrinter (Qualicon Inc., Wilmington, Del.), as previously described (6), with bacterial isolates grown overnight on BHI agar. Template preparation, restriction enzyme digestion, gel electrophoresis, and Southern hybridization probing with an *Escherichia coli* *rrnB* rRNA operon probe were carried out with the RiboPrinter system. Images were acquired with a charge-coupled-device camera and processed using the RiboPrinter's custom software. This software normalizes fragment pattern data for the band intensity and relative band position (6). Similarity coefficients for ribotype patterns from different isolates were calculated using the RiboPrinter's proprietary algorithm. Similarity coefficients and visual evaluation were used to define distinct ribotypes.

**PCR and DNA sequencing.** While traditional MLST schemes focus on the sequencing of housekeeping genes, which diversify slowly and are not heavily influenced by evolutionary forces other than point mutations (i.e., positive selection and recombination) (31), we chose to include a secreted and putative virulence gene (*hylB*) as a target for DNA sequencing-based subtyping, since the sequencing of virulence genes often provides higher discriminatory power and can provide insight into the evolution of virulence-related characteristics (7). Cell lysates for PCR were prepared using lysozyme and proteinase K as described previously (13). Primers Sa-hylF (5'-CAT ACC TTA ACA AAG ATA TAT AAC CCA AA-3') and Sa-hylR (5'-AGA TTT TTT AGA GAA TGA GAA GTT TTT T-3') were designed from known sequences (GenBank accession numbers Y15903 and U15050) to amplify a 950-bp fragment of *hylB*. Primers sodA-F (5'-GCC CTA ATT AAA GAA ACA AAA GAG T-3') and sodA-R (5'-CTG TTA TCA GCT TGC TTA CCT TAA A-3') were designed to amplify a 911-bp fragment that included the complete *sodA* coding region (609 bp). Primers sodA-F2 (5'-GAT TTA GCC TTA TTA AAA ATT GCT CAT-3') and sodA-R2 (5'-ATT CGC TTA ATT GAA GAT AAT AGG ACT GT-3') were used to amplify an 849-bp fragment for three bovine isolates (FSL S3-038, FSL S3-066, and FSL S3-081), which showed no amplification product with sodA-F

and *sodA*-R. *hylB* and *sodA* PCR conditions consisted of an initial 5-min denaturation step at 95°C, 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and a final 10-min extension cycle at 72°C. PCR products were purified using the QIAquick PCR purification kit (QIAGEN, Inc., Chatsworth, Calif.). Following gel electrophoresis, DNA was quantified by using the LabImage gel analysis software (version 2.62; Kapelan, Saale, Germany) to compare the band intensities of PCR products to marker band intensities of known concentrations (pGEM marker; Promega, Madison, Wis.).

The sequencing of PCR products was performed at Cornell University's BioResource Center by using the respective PCR primers, Big Dye Terminator chemistry, and AmpliTaq-FS DNA polymerase; the sequencing reactions were run on the ABI 3700 automated sequencer (Perkin-Elmer Biosystems, Foster City, Calif.). DNA sequence data obtained with both forward and reverse primers were proofread and assembled with the Seqman software program (DNA Star, Madison, Wis.) to yield the final *hylB* and *sodA* sequences used for analyses.

**DNA sequence analysis.** *hylB* and *sodA* sequences were aligned using the Clustal W algorithm in MegAlign (DNASTar). The *sodA* alignments used for the analyses described below included the full *sodA* coding region (609 bp); *hylB* alignments encompassed an 886-bp *hylB* fragment. Alignments were used to assign *hylB* and *sodA* alleles; individual gene sequences that differed by a single nucleotide were assigned an allelic type (e.g., *hyl-1.0*, *hyl-1.1*, or *hyl-3.0*). Allelic types were designated so that groups of closely related allelic types that differed from each other by single nucleotides were assigned decimals of the same number (e.g., *hyl-1.0* and *hyl-1.1*).

The TOPAL software program (33) was used to probe for indications of intragenic recombination events. Neighbor-joining phylogenetic trees were generated with MEGA software version 2.1 (28). Phylogenetic trees based on maximum parsimony with bootstrapped sequence data were constructed using the SEQBOOT, DNAPARS, CONSENSE, and DRAWTREE programs of the PHYLIP version 3.5c software package (12). Phylogenetic trees were also used to categorize closely related *sodA* and *hylB* allelic types into clusters.

The  $d_N/d_S$  (the number of nonsynonymous substitutions per nonsynonymous site)/ $d_S$  (the number of synonymous substitutions per synonymous site) ratios for *hylB* and *sodA* were also calculated using MEGA version 2.1 (28). The LIAN 3.0 software program (17) was used to perform a significance test for linkage disequilibrium among subtypes generated by ribotyping, *hylB* allelic typing, and *sodA* allelic typing.

**Simpson's index of discrimination.** Simpson's numerical index of discrimination (SID) provides an indication of the discriminatory power of a given subtyping method (21), as well as an estimate of the subtype diversity within a given population (16). By use of a method described by Hunter and Gaston (21), the SID was calculated in order to determine the discriminatory power of the subtyping methods used. The 95% confidence intervals surrounding the corresponding indices were calculated using the method described by Grundmann et al. (16).

**Microplate assay for hyaluronidase activity.** Hyaluronidase activities were determined for a set of 43 *S. agalactiae* isolates, representative of the genetic diversity of our original set of 104 isolates. Isolates were specifically selected to encompass all EcoRI ribotypes and *hylB* clusters represented in our isolate set. Hyaluronic acid (from human umbilical cord), cetylpyridinium chloride, and sodium azide were all purchased from Sigma Chemical Co. (St. Louis, Mo.). To measure hyaluronidase activity, *S. agalactiae* isolates were grown overnight in BHI broth at 37°C without shaking. Hyaluronidase activity was determined according to a method previously described by Tung et al. (44), with some modifications. Hyaluronic acid (5 mg/ml) was dissolved in water overnight at 4°C and then stored at -20°C. A 1% (wt/vol) sodium azide solution was prepared in water and stored at room temperature. Agarose was dissolved in 0.3 M sodium phosphate buffer (pH 7.0) in a microwave oven and maintained at 55°C before use. Hyaluronic acid and sodium azide solutions were preheated to 55°C and mixed with the agarose to give final concentrations of 0.8 mg of hyaluronic acid/ml, 0.1% (wt/vol) sodium azide, and 0.8% (wt/vol) agarose. The warm mixture was dispensed in 100- $\mu$ l aliquots into each well of a 96-well microtiter plate. Once the gel solidified, each well was filled with 100  $\mu$ l of a 1:20 dilution of *S. agalactiae* broth culture or 100  $\mu$ l of sodium phosphate as a blank. After incubation at 37°C for 18 h, the supernatant was removed from each well. Wells were washed three times with 150  $\mu$ l of sodium phosphate buffer and then filled with 100  $\mu$ l of 10% (wt/vol) aqueous cetylpyridinium chloride to precipitate the hyaluronic acid. After incubation at room temperature for 2 h, the absorbance at 600 nm was measured using a Fusion automated microplate reader (Perkin-Elmer). Absorbance values were standardized using the formula  $1 - (A_{\text{sample}}/A_{\text{blank}})$ , where  $A_{\text{sample}}$  is the absorbance of the culture sample and  $A_{\text{blank}}$  is the absorbance of the blank. The enzyme activity for each bacterial isolate was determined in duplicate by measuring the absorbance for two wells loaded with

aliquots of the same *S. agalactiae* broth culture. The assay was replicated twice for each isolate.

**Tissue culture assays.** Three human isolates and three bovine isolates representative of the most common *S. agalactiae* *hylB* allelic types (*hyl-3.0*, *hyl-4.0*, and *hyl-7.0* for human isolates and *hyl-1.0*, *hyl-5.0*, and *hyl-6.0* for bovine isolates) (Table 1) were tested for their invasion phenotypes in HeLa cells. Invasion assays were performed as previously described (40) with minor modifications. Briefly, *S. agalactiae* isolates were passaged twice to a final optical density at 600 nm of 0.4 in BHI broth. Bacterial cells were inoculated (at approximately  $10^6$  CFU per well) onto a monolayer of HeLa cells grown on coverslips in six-well plates with Dulbecco's modified Eagle medium (Invitrogen Co., Carlsbad, Calif.) and 10% fetal bovine serum. Six-well plates were then centrifuged at  $800 \times g$  for 10 min at 25°C to bring *S. agalactiae* cells into contact with the HeLa cell monolayer. Plates were allowed to incubate for 2 h at 37°C in 5% CO<sub>2</sub>. Cells were then washed three times with phosphate-buffered saline, followed by addition of Dulbecco's modified Eagle medium containing penicillin (5  $\mu$ g/ml) and gentamicin (100  $\mu$ g/ml). Infected cells were incubated for another 2 h at 37°C in 5% CO<sub>2</sub> to kill extracellular bacterial cells. Coverslips were then removed, lysed in 0.1% Triton X-100, and vortexed for 30 s. The number of CFU invaded per coverslip for each *S. agalactiae* isolate was determined by plating serial dilutions on BHI agar. Four independent repetitions of this invasion assay were performed.

**Statistical methods.** For all statistical analyses, isolates with closely related *hylB* and *sodA* allelic types were grouped into *hylB* and *sodA* clusters, which included closely related alleles as determined by phylogenetic analyses (see below). The frequency distributions of each genetic characteristic (i.e., ribotype, *hylB* cluster, or *sodA* cluster) for human and bovine isolates were compared by using a  $\chi^2$  test of independence. For comparisons in which one or more of the expected values were <5, Fisher's exact test was performed using the SAS program (version 8.01; SAS Institute, Cary, N.C.). *P* values of <0.05 were considered statistically significant.

To determine associations between either the host species or the *hylB* cluster and hyaluronidase activity, a nonparametric analysis of variance (Kruskal-Wallis) was conducted with the Statistix software program (Analytical Software, Tallahassee, Fla.). Nonparametric analyses were deemed appropriate, as the activity data deviated significantly from normality. Associations were analyzed based on averaged assay results for each isolate. Data for isolates in clusters *hyl-7* and *hyl-2* were excluded from the analysis of association between *hylB* clusters and activity levels because these two clusters were represented by two isolates and one isolate only, respectively, in the hyaluronidase activity assays.

To determine whether human and bovine strains differed significantly in their abilities to invade HeLa cells, a one-way analysis of variance was conducted using the Minitab software program (version 14; Minitab, Inc., State College, Pa.).

**World Wide Web-based data access.** Detailed isolate source information and raw data from this study (*hylB* and *sodA* sequences and EcoRI ribotype patterns) are accessible through the PathogenTracker version 2.0 website (<http://www.pathogentracker.net>).

## RESULTS

**Ribotype pattern analysis.** A total of 17 distinct EcoRI ribotypes were identified among the 104 human and bovine *S. agalactiae* isolates characterized (Table 1 and Fig. 2). Human isolates clustered into four ribotypes; ribotype 106-1501-S-8 represented the majority of human isolates (75%). All four EcoRI ribotypes that contained human isolates also contained bovine isolates; however, 13 ribotypes contained bovine isolates exclusively. Eight ribotypes contained only one bovine isolate each. A two-tailed Fisher's exact test demonstrated significant associations between five ribotypes and isolation from either human or bovine hosts (Table 1).

***hylB* allelic and phylogenetic analysis.** Initial analyses using TOPAL (33) revealed no indications of recombination events in the 886-bp *hylB* fragment analyzed. A total of 17 allelic types were found among the 104 *S. agalactiae* isolates characterized. A neighbor-joining tree constructed by using two representatives of each *hylB* allelic type (where available) revealed the phylogenetic relationships among allelic types and was used to

TABLE 1. Frequency distribution of *S. agalactiae* isolates among EcoRI ribotypes, *hylB* allelic types, and *sodA* allelic types

Subtype	Incidence (prevalence [%]) among indicated type of isolates		P value <sup>a</sup>
	Human (n = 52)	Bovine (n = 52)	
<b>EcoRI ribotype</b>			
106-1501-S-8	39 (75.0)	1 (1.9)	<0.01
116-549-S-1	7 (13.5)	3 (5.8)	0.50
106-1501-S-5	4 (7.7)	12 (23.1)	0.01
116-611-S-7	2 (3.8)	3 (5.8)	0.65
106-1502-S-6	0	9 (17.3)	<0.01
106-1502-S-2	0	6 (11.5)	0.01
106-1502-S-3	0	4 (7.7)	0.03
106-1504-S-1	0	3 (5.8)	0.08
116-522-S-1	0	2 (3.8)	NA
106-1501-S-4	0	2 (3.8)	NA
116-549-S-7	0	1 (1.9)	NA
116-574-S-2	0	1 (1.9)	NA
116-607-S-6	0	1 (1.9)	NA
116-622-S-8	0	1 (1.9)	NA
116-638-S-1	0	1 (1.9)	NA
116-646-S-2	0	1 (1.9)	NA
106-1502-S-1	0	1 (1.9)	NA
<b><i>hylB</i> clusters and allelic types<sup>b</sup></b>			
<i>hyl</i> -1 cluster	0	31 (59.6)	<0.01
<i>hyl</i> -1.0	0	24 (46.2)	
<i>hyl</i> -1.1	0	5 (9.6)	
<i>hyl</i> -1.2	0	1 (1.9)	
<i>hyl</i> -1.3	0	1 (1.9)	
<i>hyl</i> -2 cluster ( <i>hyl</i> -2.0)	2 (3.8)	0	0.50
<i>hyl</i> -3 cluster ( <i>hyl</i> -3.0)	7 (13.5)	0	0.01
<i>hyl</i> -4 cluster ( <i>hyl</i> -4.0)	26 (50.0)	1 (1.9)	<0.01
<i>hyl</i> -5 cluster	0	17 (32.6)	<0.01
<i>hyl</i> -5.0	0	13 (25.0)	
<i>hyl</i> -5.1	0	1 (1.9)	
<i>hyl</i> -5.2	0	1 (1.9)	
<i>hyl</i> -5.3	0	1 (1.9)	
<i>hyl</i> -5.4	0	1 (1.9)	
<i>hyl</i> -6 cluster	9 (17.3)	3 (5.7)	0.12
<i>hyl</i> -6.0	7 (13.5)	2 (3.8)	
<i>hyl</i> -6.1	2 (3.8)	0	
<i>hyl</i> -6.2	0	1 (1.9)	
<i>hyl</i> -7 cluster	8 (15.4)	0	0.01
<i>hyl</i> -7.0	7 (13.5)	0	
<i>hyl</i> -7.1	1 (1.9)	0	
<b><i>sodA</i> clusters and allelic types<sup>b</sup></b>			
<i>sod</i> -1 cluster	41 (78.8)	5 (9.6)	<0.01
<i>sod</i> -1.0	40 (76.9)	4 (7.7)	
<i>sod</i> -1.1	1 (1.9)	0	
<i>sod</i> -1.2	0	1 (1.9)	
<i>sod</i> -2 cluster ( <i>sod</i> -2.0)	3 (5.8)	0	0.24
<i>sod</i> -3 cluster ( <i>sod</i> -3.0)	6 (11.5)	0	0.03
<i>sod</i> -4 cluster	2 (3.8)	47 (90.4)	<0.01
<i>sod</i> -4.0	2 (3.8)	46 (88.5)	
<i>sod</i> -4.1	0	1 (1.9)	

<sup>a</sup> All P values (between human and bovine isolates) were computed using a two-tailed Fisher's exact test. NA, Fisher's exact test was not applicable.

<sup>b</sup> P values were computed for *hylB* and *sodA* clusters only (e.g., *hyl*-1, *hyl*-2, *sod*-1, or *sod*-2).

group *hylB* allelic types into seven *hylB* clusters, which included closely related *hylB* alleles (Fig. 3). A parsimony tree created by using DNAPARS in the PHYLIP program showed topology and clustering of human and bovine isolates similar to those of

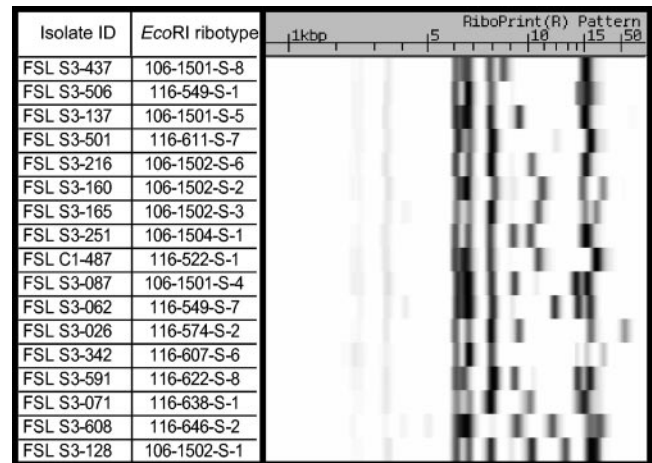


FIG. 2. EcoRI ribotype patterns obtained for human and bovine *S. agalactiae* isolates. The left-hand side of the figure includes the identification codes (ID; e.g., FSL S3-437) of the isolates representative of the 17 EcoRI ribotypes encountered, along with the corresponding ribotype designations (e.g., 106-1501-S-8). Ribotype patterns are shown on the right-hand side and correspond to a gel run from right to left (high molecular weight bands are thus shown at the far right). Ribotype patterns for all 104 isolates can be viewed at the PathogenTracker website (<http://www.pathogentracker.net>).

the neighbor-joining phylogenetic tree (not shown). A two-tailed Fisher's exact test demonstrated significant associations between five *hylB* clusters and isolation from either human or bovine hosts (Table 1). Statistical analyses were not conducted for each *hylB* allelic type, due to the small numbers of isolates for some allelic types (Table 1).

PCR amplification of *hylB* revealed a PCR product approximately 1,300 bp larger than expected for 8 of the 52 human isolates and none of the 52 bovine isolates. DNA sequencing of these PCR products revealed a putative insertion sequence within *hylB*, which showed >98% homology with IS1548 according to the basic local alignment search tool (1). The IS1548 insertion was mapped to the same *hylB* location in all eight isolates. All eight human isolates with the IS1548 insertion in *hylB* share the same ribotype (106-1501-S-8). After the removal of the IS1548 sequence, the *hylB* sequences for these eight isolates were included in the overall *hylB* alignment for allele assignment and phylogenetic analysis. All eight isolates were shown to share the same *hylB* allele (*hyl*-7.0), with the exception of one isolate (FSL S3-003), which is the only isolate with allele *hyl*-7.1.

To further probe the evolution of the *hylB* fragments sequenced among human and bovine isolates, the  $d_N/d_S$  ratios for the sequenced fragments were calculated. The  $d_N/d_S$  ratio obtained for human isolates (0.425) was slightly higher than the ratio obtained for the bovine isolates (0.357). The overall  $d_N/d_S$  ratio for *hylB* was 0.382. A total of 39 polymorphic nucleotide sites were found among the 52 human isolates, including 27 sites with nonsynonymous substitutions. Among the bovine isolates, there were 40 polymorphic sites, including 23 sites with nonsynonymous substitutions.

***sodA* allelic analysis.** A total of seven different *sodA* allelic types were identified (Table 1). Based on phylogenetic analysis, these types could be grouped into four *sodA* clusters, which

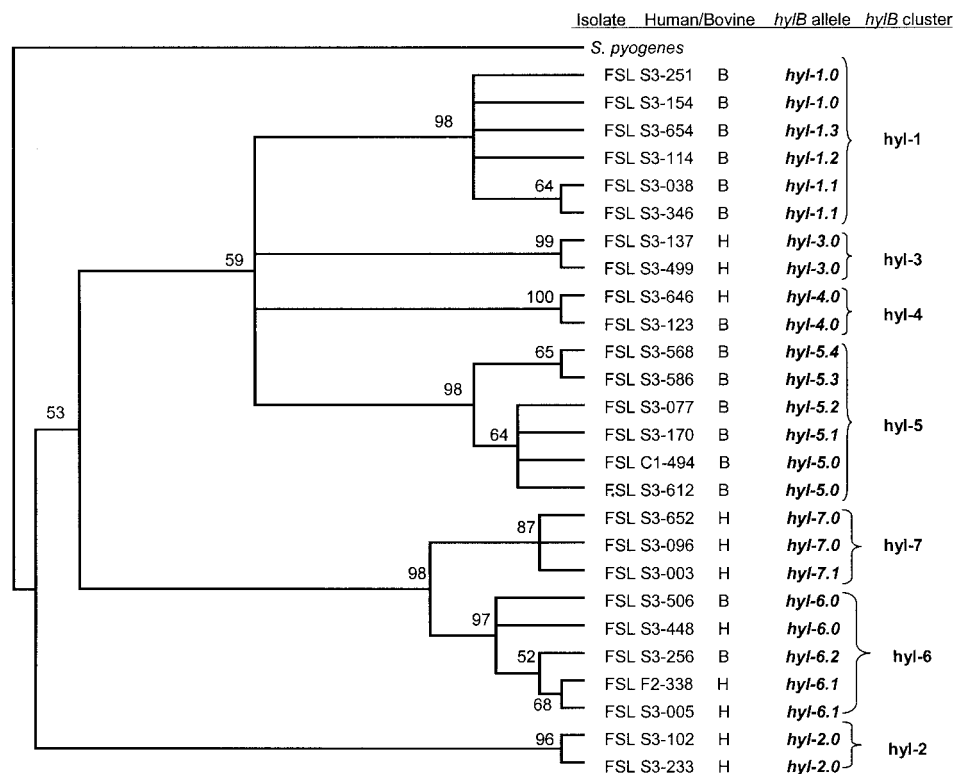


FIG. 3. Consensus neighbor-joining tree based on partial *hylB* sequences. Two representative sequences of each *hylB* allelic type (where available) were used for the construction of this phylogenetic tree. An *S. pyogenes hylA* sequence (encoding hyaluronate lyase) was used as an outgroup (GenBank accession number NC002737). The tree was built by using the Jukes-Cantor model of nucleotide substitution. A total of 1,000 bootstrap replicates were generated. Bootstrap values of >50% are shown at the nodes. Closely related *hylB* allelic types were assigned an *hylB* cluster (e.g., cluster hyl-2), which is also indicated in this figure.

contain allelic types that differ by single-nucleotide differences. The majority of human isolates (76.9%) clustered into allelic type *sod-1.0* and cluster *sod-1*, while the majority of bovine isolates (88.5%) clustered into allelic type *sod-4.0* and cluster *sod-4*. As a result, these *sod* clusters (*sod-1* and *sod-4*) were significantly host associated ( $P < 0.01$  for both alleles) (Table 1).

Direct nucleotide sequence analysis of the complete *sodA* coding sequence revealed limited diversity among the 104 *S. agalactiae* isolates characterized. Within the 609-nucleotide coding sequence, a total of 12 polymorphic nucleotide sites were identified. The  $d_N/d_S$  ratio for *sodA* was 0.250 (0.333 and 0.200 for human and bovine isolates, respectively).

**Distribution of *S. agalactiae* clonal groups among human and bovine isolates.** The standardized index of association ( $I_A$ ), a measure of linkage disequilibrium (17), was calculated based on subtypes generated by the three subtyping methods for all 104 *S. agalactiae* isolates. The standardized  $I_A$  was calculated as 0.41, which represents a highly significant ( $P < 0.0001$ ) indication of linkage disequilibrium (and, thus, of a clonal population structure) among the isolates characterized.

Clonal groups were defined as unique combinations of subtypes (EcoRI ribotypes and *hylB* and *sodA* allelic types) as previously proposed by van Belkum et al. (45). A total of 39 clonal groups were found among the 104 human and bovine isolates (Table 2). Eleven clonal groups were unique to human

isolates, and 26 clonal groups were unique to bovine isolates. Only two clonal groups shared both human and bovine isolates; clonal group A included 25 human isolates and 1 bovine isolate, and clonal group G included 1 human isolate and 1 bovine isolate.

**Discriminatory power of subtyping methods and genetic diversity.** The SIDs, along with the corresponding confidence intervals, were determined for all subtyping methods (EcoRI ribotyping and *hylB* and *sodA* allelic types) across three populations of isolates (human only, bovine only, and human and bovine) (Table 3). The combination of all three methods allowed for an index of discrimination (SID [mean  $\pm$  standard deviation],  $0.93 \pm 0.03$ ) that was almost identical to the SID obtained by EcoRI ribotyping and *hylB* sequence typing combined ( $0.92 \pm 0.03$ ). The SID obtained for EcoRI ribotyping alone, as well as that for EcoRI ribotyping combined with *hylB* and/or *sodA* sequencing, showed a significantly higher genetic diversity among bovine isolates than among human isolates (Table 3).

**Hyaluronidase activity among selected *S. agalactiae* isolates.** Hyaluronidase activities were determined for 43 representative *S. agalactiae* isolates. The average hyaluronidase activity of human isolates ( $n = 15$ ) was significantly lower than the average hyaluronidase activity for bovine isolates ( $n = 28$ ) ( $P < 0.01$ ). Frequency distributions of average hyaluronidase activities for *hylB* clusters (Fig. 4) revealed that most isolates in

TABLE 2. Genetic characteristics and distributions of *S. agalactiae* clonal groups

Clonal group	EcoRI ribotype	Allele of indicated type		Incidence in indicated type(s) of isolates		
		<i>hylB</i>	<i>sodA</i>	Human ( <i>n</i> = 52)	Bovine ( <i>n</i> = 52)	Human and Bovine ( <i>n</i> = 104)
A	106-1501-S-8	<i>hyl</i> -4.0	<i>sod</i> -1.0	24	1	25
B	106-1501-S-8	<i>hyl</i> -7.0	<i>sod</i> -1.0	7	0	7
C	116-549-S-1	<i>hyl</i> -6.0	<i>sod</i> -3.0	6	0	6
D	106-1501-S-8	<i>hyl</i> -3.0	<i>sod</i> -1.0	4	0	4
E	106-1501-S-8	<i>hyl</i> -2.0	<i>sod</i> -2.0	2	0	2
F	116-611-S-7	<i>hyl</i> -3.0	<i>sod</i> -1.0	2	0	2
G	116-549-S-1	<i>hyl</i> -6.0	<i>sod</i> -1.0	1	1	2
H	106-1501-S-5	<i>hyl</i> -3.0	<i>sod</i> -1.0	1	0	1
I	106-1501-S-5	<i>hyl</i> -4.0	<i>sod</i> -4.0	1	0	1
J	106-1501-S-5	<i>hyl</i> -6.1	<i>sod</i> -2.0	1	0	1
K	106-1501-S-5	<i>hyl</i> -6.1	<i>sod</i> -4.0	1	0	1
L	106-1501-S-8	<i>hyl</i> -4.0	<i>sod</i> -1.1	1	0	1
M	106-1501-S-8	<i>hyl</i> -7.1	<i>sod</i> -1.0	1	0	1
N	106-1502-S-6	<i>hyl</i> -1.0	<i>sod</i> -4.0	0	7	7
O	106-1501-S-5	<i>hyl</i> -1.0	<i>sod</i> -4.0	0	5	5
P	106-1501-S-5	<i>hyl</i> -1.1	<i>sod</i> -4.0	0	5	5
Q	106-1502-S-2	<i>hyl</i> -5.0	<i>sod</i> -4.0	0	5	5
R	106-1502-S-3	<i>hyl</i> -5.0	<i>sod</i> -4.0	0	3	3
S	106-1504-S-1	<i>hyl</i> -1.0	<i>sod</i> -4.0	0	3	3
T	106-1501-S-4	<i>hyl</i> -1.0	<i>sod</i> -4.0	0	2	2
U	116-611-S-7	<i>hyl</i> -1.0	<i>sod</i> -4.0	0	2	2
V	106-1501-S-5	<i>hyl</i> -1.2	<i>sod</i> -4.0	0	1	1
W	106-1501-S-5	<i>hyl</i> -5.0	<i>sod</i> -4.0	0	1	1
X	106-1502-S-1	<i>hyl</i> -1.0	<i>sod</i> -4.0	0	1	1
Y	106-1502-S-2	<i>hyl</i> -5.1	<i>sod</i> -4.0	0	1	1
Z	106-1502-S-3	<i>hyl</i> -5.4	<i>sod</i> -4.0	0	1	1
AA	106-1502-S-6	<i>hyl</i> -5.0	<i>sod</i> -1.2	0	1	1
AB	106-1502-S-6	<i>hyl</i> -5.0	<i>sod</i> -4.0	0	1	1
AC	116-522-S-1	<i>hyl</i> -5.0	<i>sod</i> -4.0	0	1	1
AD	116-522-S-1	<i>hyl</i> -5.2	<i>sod</i> -4.0	0	1	1
AE	116-549-S-1	<i>hyl</i> -1.3	<i>sod</i> -4.1	0	1	1
AF	116-549-S-1	<i>hyl</i> -6.2	<i>sod</i> -1.0	0	1	1
AG	116-549-S-7	<i>hyl</i> -1.0	<i>sod</i> -4.0	0	1	1
AH	116-574-S-2	<i>hyl</i> -5.0	<i>sod</i> -4.0	0	1	1
AI	116-607-S-6	<i>hyl</i> -6.0	<i>sod</i> -1.0	0	1	1
AJ	116-611-S-7	<i>hyl</i> -5.3	<i>sod</i> -4.0	0	1	1
AK	116-622-S-8	<i>hyl</i> -1.0	<i>sod</i> -4.0	0	1	1
AL	116-638-S-1	<i>hyl</i> -1.0	<i>sod</i> -4.0	0	1	1
AM	116-646-S-2	<i>hyl</i> -1.0	<i>sod</i> -4.0	0	1	1

cluster *hyl*-1 had activities towards the upper range of the data, whereas isolates in clusters *hyl*-3 and *hyl*-4 were distributed across the lower and middle ranges of the data, respectively. Clusters *hyl*-5 and *hyl*-6 showed evidence of a bimodal distribution of activities; some isolates had no activity, while others had medium to high activity. There was no apparent correlation between *hyl* alleles (e.g., *hyl*-5.1 and *hyl*-5.2) and hyaluronidase activities, but no formal statistical analyses for associations could be conducted, due to the small number of isolates

assayed for each suballele. All isolates in cluster *hyl*-7, which contained insertions of the transposable element IS1548 in *hylB*, showed no hyaluronidase activity. In addition, two bovine isolates and one human isolate in cluster *hyl*-6 and two animal isolates in cluster *hyl*-5 also showed no detectable hyaluronidase activity in this in vitro assay. Based on the Kruskal-Wallis analysis of variance, there is a statistically significant difference in hyaluronidase activity between *hylB* clusters. Specifically, isolates in cluster *hyl*-1, which is associated with bovine hosts, showed a significantly higher average level of hyaluronidase activity than isolates in cluster *hyl*-3, which is associated with human hosts, and isolates in cluster *hyl*-6, which is numerically but not statistically associated with human hosts. Activities for isolates in clusters *hyl*-4 and *hyl*-5 overlap with those for isolates in cluster *hyl*-1 (the high end of the activity range for both cluster *hyl*-4 and cluster *hyl*-5) as well as with activities for isolates in clusters *hyl*-3 and *hyl*-6 (the low end of the activity range for both cluster *hyl*-4 and cluster *hyl*-5).

**Tissue culture invasion assay.** Representative human (*n* = 3) and bovine (*n* = 3) *S. agalactiae* isolates were evaluated for their invasion phenotypes in the HeLa human epithelial cell line. There was no significant difference in relative invasions of human and bovine isolates (*P* = 0.5; one-way analysis of variance) (Fig. 5). One of the isolates tested (FSL S3-008) contained allele *hyl*-7.0, which is characterized by an IS1548 insertion in *hylB*. This isolate did not differ from the other isolates, which did not carry an IS1548 insertion in the sequenced *hylB* fragment, in HeLa invasion properties.

## DISCUSSION

While it has been well established that bacteria classified in the species *S. agalactiae* can cause severe human invasive disease as well as clinical and subclinical mastitis in cattle, the likelihood and potential of *S. agalactiae* transmission between these two host species has not been clearly defined. Based on our data, we conclude that (i) EcoRI ribotyping in combination with *hylB* and *sodA* sequencing provides for discriminatory subtype analysis of *S. agalactiae*; (ii) human invasive and bovine *S. agalactiae* strains represent distinct populations with limited interspecies transmission, as determined by both molecular subtype data and phenotypic data (i.e., hyaluronidase activities); and (iii) hyaluronidase activity is not required for all human infections.

**EcoRI ribotyping in combination with *hylB* and *sodA* sequencing provides a discriminatory subtyping approach for *S.***

TABLE 3. SIDs for different *S. agalactiae* subtyping methods

Method(s)	SID (95% confidence interval) for indicated type(s) of isolates		
	Human ( <i>n</i> = 52)	Bovine ( <i>n</i> = 52)	Human and bovine ( <i>n</i> = 104)
EcoRI ribotyping	0.42 (0.27, 0.57)	0.90 (0.85, 0.94)	0.81 (0.75, 0.87)
<i>hylB</i> allelic typing	0.71 (0.60, 0.82)	0.73 (0.64, 0.81)	0.85 (0.82, 0.89)
<i>sodA</i> allelic typing	0.40 (0.23, 0.56)	0.22 (0.07, 0.36)	0.61 (0.56, 0.66)
EcoRI ribotyping and <i>sodA</i> allelic typing	0.51 (0.35, 0.67)	0.91 (0.86, 0.95)	0.84 (0.79, 0.90)
EcoRI ribotyping and <i>hylB</i> allelic typing	0.74 (0.63, 0.84)	0.96 (0.94, 0.98)	0.92 (0.88, 0.95)
<i>hylB</i> allelic typing and <i>sodA</i> allelic typing	0.75 (0.65, 0.85)	0.73 (0.63, 0.83)	0.87 (0.83, 0.90)
EcoRI ribotyping and <i>hylB</i> allelic typing and <i>sodA</i> allelic typing	0.76 (0.65, 0.87)	0.96 (0.94, 0.97)	0.93 (0.89, 0.96)

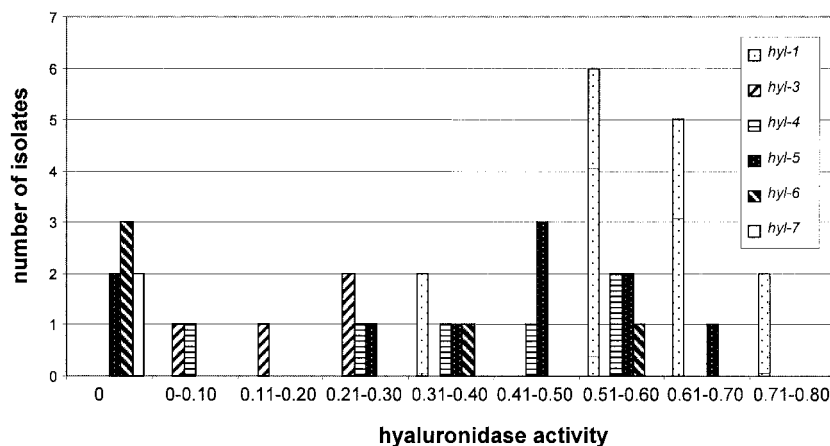


FIG. 4. Frequency distribution of hyaluronidase activity levels for isolates grouping into different *hylB* clusters. Labels on the x axis indicate activities higher than the lower bound of the range and up to and including the upper bound of the range. Hyaluronidase activity levels are expressed as absorbance values normalized to the negative control absorbance values; higher values indicate higher levels of hyaluronidase activity.

*agalactiae*. While banding pattern-based DNA subtyping methods (e.g., RAPD analysis) have been shown to successfully differentiate among *S. agalactiae* strains (32), difficulties in standardization and reproducibility of results can limit the usefulness of some of these subtyping methods. DNA sequencing-based subtyping methods, on the other hand, provide highly standardized and portable subtype data, which are also suitable for phylogenetic analyses (31). Thus, we used automated EcoRI ribotyping, one of the most standardized and reproducible DNA banding-based subtyping methods currently available (6, 47), and DNA sequencing of the putative virulence gene *hylB* (encoding hyaluronidase) and the housekeeping gene *sodA* (encoding superoxide dismutase) as subtyping methods for *S. agalactiae* in this study. Ribotyping has been applied to various species and has been reported to be useful for population genetics and transmission studies be-

cause this method scores evolutionary changes that appear to accumulate slowly enough to allow large-scale surveillance yet occur frequently enough to provide sufficient discrimination to track epidemic clones (43). DNA sequencing-based subtyping methods, on the other hand, produce not only highly reproducible and unambiguous data but also, unlike banding-based DNA subtyping methods, provide data suitable for evolutionary analysis (47).

While our data showed that EcoRI ribotyping or allelic analysis of *sodA* or *hylB* alone provided only limited subtype discrimination, the combination of the different subtyping methods provided sufficient discrimination for our studies. Interestingly, *sodA*, which encodes a superoxide dismutase (14), appeared highly conserved and of limited subtype diversity, while *hylB*, a putative virulence gene (30), showed significantly higher diversity. These data further support our choice of including the sequencing of a putative virulence gene (*hylB*) in a DNA sequencing-based typing scheme and are consistent with previous observations that the sequencing of virulence genes provides higher subtype discrimination than the sequencing of housekeeping genes (7). Sequencing data from housekeeping genes are necessary for long-term epidemiological analyses, though, since these genes are unlikely to be under positive selection (31, 42). In a previous study, ribotyping using enzymes PstI, HindIII, and CfoI was able to differentiate between unrelated *S. agalactiae* isolates from neonates, with an SID of >0.95 when data for different enzymes were combined; single-enzyme ribotyping provided discrimination similar to that reported here for EcoRI ribotyping (8). EcoRI ribotyping provided easily interpretable and distinct patterns (Fig. 2), and we thus conclude that the combination of EcoRI ribotyping and allelic analysis of *sodA* or *hylB* provides a suitable subtyping approach for studying the transmission and epidemiology of human and bovine *S. agalactiae* isolates.

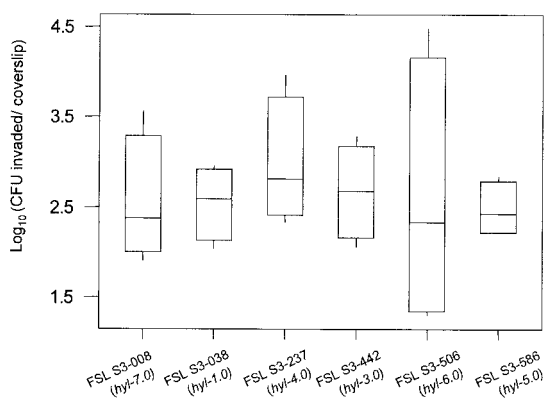


FIG. 5. Box plot of HeLa cell invasion efficiency of three human *S. agalactiae* isolates and three bovine *S. agalactiae* isolates. Invasion phenotypes as shown on the y axis were measured as log<sub>10</sub> (CFU invaded per coverslip). Values represent the medians and interquartile ranges from four independent experiments. Isolates tested are indicated on the x axis; isolates FSL-S3-008, FSL-S3-237, and FSL-S3-442 represent isolates from humans with invasive infections, and isolates FSL-S3-038, FSL-S3-506, and FSL-S3-586 were obtained from bovine hosts.

**Human and bovine *S. agalactiae* isolates represent distinct subtypes and populations.** Studies on transmission and epidemiology require sensitive and reproducible subtyping approaches, as well as truly representative and unbiased isolate collections appropriate for the hypothesis tested. Our hypoth-

esis was that bovine *S. agalactiae* isolates in milk represent distinct subtypes from those *S. agalactiae* isolates that cause human invasive disease. To test this hypothesis, we conducted a prospective study of temporally and geographically matched *S. agalactiae* isolates collected through existing surveillance systems in New York. While many previous subtyping studies on human *S. agalactiae* strains have used and/or included isolates from asymptomatic carriers (4, 18, 32, 38), the human isolates used here were exclusively from human invasive infections. Molecular subtyping data for the human clinical and bovine isolates characterized here were used in two separate and independent statistical analyses (i.e., a frequency distribution of subtypes and a comparison of the genetic diversities of human and bovine populations) to probe whether human clinical isolates and bovine milk isolates represent separate populations.

Each of the three subtyping approaches used clearly provided strain discrimination into subtypes that largely showed host-specific distribution patterns. Although associations were not always exclusive, our data revealed several statistically significant and exclusive associations between subtypes and host species. Utilizing an approach proposed by van Belkum et al. (45), we also used a combination of all the subtyping methods to define *S. agalactiae* clonal groups. Human and bovine isolates largely grouped into separate clonal groups; only 2 of the 39 clonal groups contained both human and bovine isolates. These data are consistent with an RAPD subtyping study on 223 *S. agalactiae* isolates from bovines and human asymptomatic carriers (32), which found that human and bovine *S. agalactiae* isolates largely represented separate subtypes, even though one human isolate and one bovine isolate had identical RAPD types. Our data are also consistent with a recent MLST study on bovine and human invasive and carrier isolates, which showed that human and bovine isolates represent largely separate populations and in which only 3 of 50 sequence types were found in both humans and cattle (4). While this study found that a single hyperinvasive clonal group linked to neonatal infections grouped with bovine isolates and has likely arisen from a bovine ancestor, this clonal group was unique to humans and was not isolated from any bovine cases (4).

A comparison of the genetic diversities (as determined by EcoRI ribotyping) of human and bovine *S. agalactiae* populations performed by using Simpson's index of diversity as described by Grundmann et al. (16) showed that these *S. agalactiae* isolates represent distinct populations. Interestingly, bovine isolates showed a significantly higher EcoRI ribotype diversity than human isolates, while *hylB* and *sodA* allelic diversities did not differ significantly between human and bovine isolates. The relatively low degree of genetic diversity among human isolates is consistent with previous studies, which showed that *S. agalactiae* isolates from humans with invasive infections had less diversity than isolates from asymptomatic human carriers, which had a high degree of genetic diversity (19, 32). While a recent MLST study showed that human isolates were more diverse than bovine isolates, human isolates in this study were obtained from multiple countries and continents and represented both patients with invasive cases and carriers, whereas bovine isolates were from a single country (4). While our data thus support the idea that bovine milk isolates and human invasive isolates of *S. agalactiae* represent

distinct populations with very little overlap, further studies using temporally and geographically matched isolate populations and standardized and reproducible subtyping methods (including, for example, MLST) (25) will be necessary to determine the relationships between *S. agalactiae* isolates found in human asymptomatic carriers and bovines, particularly since preliminary data provide some evidence for an overlap between these populations (24). MLST studies on temporally and geographically matched isolate populations will also be necessary to further probe the transmission of the hyperinvasive human neonatal *S. agalactiae* clone (10), which appears to have arisen from a bovine ancestor (4).

**Human and bovine isolates differ in hyaluronidase activity but not in ability to invade human epithelial cells.** Based on our preliminary *hylB* DNA sequence data and prior studies with *S. agalactiae* isolates from human patients with neonatal meningitis, which identified a single clone that produces unusually high amounts of hyaluronidase (29, 36), we focused further investigations on the role of hyaluronidase activity in *S. agalactiae* host specificity. *S. agalactiae hylB* encodes hyaluronate lyase (hyaluronidase), a putative virulence factor that is involved in cleaving hyaluronic acid, a component of the extracellular matrix in many tissues (30). Hyaluronidases have also been found to be important virulence factors in other gram-positive organisms, such as *Streptococcus pyogenes* (22). Overall, human isolates showed a significantly lower average hyaluronidase activity than bovine isolates, indicating that *S. agalactiae* isolates from cattle and human invasive cases also represent phenotypically distinct populations. Our data also show significant differences in hyaluronidase activities for *hylB* clusters, which are consistent with the host-specific distribution of *hylB* clusters that we observed. Interestingly, we found that 8 of the 52 human isolates and none of the 52 bovine isolates characterized in our study carried an IS1548 insertion in *hylB*, which was associated with a hyaluronidase-negative phenotype. Our findings are consistent with previous studies that showed the presence of the IS1548 insertion in *hylB* in *S. agalactiae* isolates from human endocarditis and septicemia patients and from human asymptomatic carriers (15) as well as in human and feline isolates collected in Europe (49). These data further support the idea that hyaluronidase may not be required for the ability of *S. agalactiae* to cause certain infections (e.g., endocarditis) in human hosts. Interestingly, we also identified an additional five isolates (including four from bovines) with a hyaluronidase-negative phenotype without an apparent IS1548 in the *hylB* fragment sequenced. This fact may indicate that hyaluronidase activity is also not always required for animal infection or colonization, or it may suggest that these strains regulate hyaluronidase activity more tightly so that it cannot be detected in vitro.

While early studies have shown that both human and bovine *S. agalactiae* isolates can adhere to bovine mammary gland epithelial cells (5), we were not able to find any prior data on the ability of bovine isolates to adhere to and invade human epithelial cells. Using an established tissue culture model of human *S. agalactiae* invasion (40), we did not find any significant difference between the invasion phenotypes of three selected bovine *S. agalactiae* isolates and three human *S. agalactiae* isolates. One isolate, which contained the IS1548 insertion in the sequenced *hylB*, did not differ from the other isolates in



its HeLa invasion properties. This fact indicates that *hylB* is not essential for the invasion of human epithelial cells.

To further probe for differences in the role of hyaluronidase in the pathogenesis of bovine mastitis and human invasive infections, we performed a preliminary analysis of the ratio of nonsynonymous nucleotide changes to synonymous nucleotide changes within the *hylB* fragment sequenced. The higher ratio for *hylB* sequences from human invasive isolates and the presence of IS1548 in only human isolates add evidence to the hypothesis of reduced functional constraints on *hylB* in human isolates compared to that in bovine isolates. While hyaluronidase appears to play an important role in the pathogenesis of bovine mammary infections, this enzyme may not be required for at least some forms of human infections and may thus have accumulated deleterious mutations (including IS1548 insertions). Similar observations have been reported for other bacterial species. For example, in *Salmonella enterica*, a loss of gene function has been observed for some serotypes that have adapted to an avian host range (3). Reductive evolution has also been documented in obligate intracellular parasites, such as *Rickettsia* and *Chlamydia* spp. (9).

**Conclusions.** Our data indicate the existence of distinct *S. agalactiae* subtypes and clonal groups that appear to differ in their host specificities as well as their pathogenic potentials. These findings provide evidence that the potential for transmission of *S. agalactiae* from a bovine host to a human host or vice versa is low. In spite of the low transmission potential between human and bovine species predicted by our results, it appears that human-associated isolates have the necessary virulence factors to occasionally cause bovine infections, as indicated by the rare occurrence of some human-associated subtypes among bovine isolates found in both this study and a recent MLST study (4). Further studies using additional human and bovine tissue culture cell lines and appropriate animal models will be necessary, though, to understand whether specific functional barriers restrict the interspecies transmission of *S. agalactiae*. Ultimately, research of this nature should lead to the development of specifically targeted disease prevention strategies for human- and bovine-associated *S. agalactiae* subtypes. Our findings also add to the increasing recognition of host-specific bacterial clones among pathogens that otherwise might be considered candidates for interspecies transmission in the absence of appropriate subtyping data (26). For example, evidence for host and/or tissue specificity among clonal groups has been described for *Pasteurella haemolytica*, which, as a species, is associated with infections of sheep and cattle (11); for *Bordetella bronchiseptica*, which is associated with infections of pigs and dogs (35); and for *Staphylococcus aureus*, which is associated with infections in human and different animal species (37).

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