Veterinary Microbiology 235 (2019) 71–79

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

The distribution of clones of *Streptococcus agalactiae* (group B streptococci) among herdspersons and dairy cows demonstrates lack of host specificity for some lineages

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ARTICLE INFO

Keywords:
Streptococcus agalactiae
Group B streptococcus
Streptococcal disease
Mastitis
Bacterial transmission
Streptococcal genetics
Streptococcal resistance
Zoonosis
Anthroponosis

ABSTRACT

Streptococcus agalactiae (Group B streptococcus, GBS) is a commensal of the human intestinal tract and vagina and is also an opportunistic pathogen causing serious, potentially lethal, infections preferentially in newborns and in the elderly. In cattle, it is considered an udder-specific pathogen and a common cause of mastitis. Here we investigated the host specificity of GBS by examining their colonization at various anatomical sites in both cattle and humans, as well as the possible cross-species transmission in closed barn environments. We collected more than 800 swab samples from dairy cows and herdspersons at eight dairy farms in Denmark. GBS was isolated from 12% of the samples. The GBS strains (N = 105) were characterized by biochemical test, serology, and Pulsed-Field Gel Electrophoresis (PFGE). Based on the PFGE patterns, 25 strains were selected for whole genome sequencing followed by phylogenetic analyses. The genomes were compared to each other and to a collection of publicly available GBS genomes. The study revealed that GBS clones were shared by cows and herdspersons. In phylogenetic analyses, these shared clones clustered with GBS strains from persons with no relation to farming. Horizontal cross-species transmission of the contagion in both directions was found to be highly likely within the same environment; thus, some cases of bovine mastitis are probably antrophonotic.

1. Introduction

Streptococcus agalactiae, referred to as group B streptococcus (GBS), is an important pathogen both in humans and a range of animal species (Delannoy et al., 2013). In humans, GBS causes serious neonatal infections (Spellerberg, 2000), invasive diseases and other infections in the elderly (Skoff et al., 2009) and in adults < 65 years (Phares et al., 2008), and it is a common cause of mastitis in dairy cattle (Lyhs et al., 2016). The infected mammary gland is the main reservoir for bovine GBS. Other reservoirs in animals are largely unknown; however, GBS were recently isolated from different body sites and from the surroundings of dairy cows (Cobo-Angel et al., 2018; Jørgensen et al., 2016). GBS is a commensal in humans with frequent reservoir both in the intestine and vagina. The bacteria may also colonize the oropharynx (de Aguiar et al., 2016; Foxman et al., 2006) and the urogenital tract in men (Foxman et al., 2006). It colonizes 15–37% of all adults in Denmark (Hansen et al., 2004) and other countries (Kwatra et al., 2016). Neonatal early onset infections are usually caused by GBS asymptomatically carried by the mother and vertically transmitted to the child during delivery (Dillon et al., 1987). The proportion of Danish dairy herds tested positive for GBS increased from less than 2% in 2000 to more than 6% in 2009 (Katholm et al., 2012; Mahmmod et al., 2013) and the same trend is reported from other Nordic countries (Katholm et al., 2012). Different evolutionary lineages of GBS are globally disseminated (Sørensen et al., 2010) and some of these (*e.g.* clonal complex 17, CC17) are associated with human infections (Teatero et al., 2016) while others (*e.g.* CC61/67) usually are related to mastitis in cattle (Sørensen et al., 2010). Although both animals and humans constitute natural reservoirs for GBS, knowledge on host specificity of this bacterium is sparse. Interestingly, GBS strains considered as genetic lineages “of human origin” have been isolated from both fish and various other aquatic animals (Delannoy et al., 2013). In 2015, an epidemic of GBS invasive infections in humans caused by sequence type ST283 was found to be related to the consumption of raw freshwater

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https://doi.org/10.1016/j.vetmic.2019.06.008
Received 17 September 2018; Received in revised form 28 May 2019; Accepted 10 June 2019

fish (Kalimuddin et al., 2017). Interspecies transmission of a GBS strain between livestock and humans has also been suggested in the past (Manning et al., 2010). Thus, under certain conditions, GBS transmission between different host species seems possible. While contaminated milking machines are regarded as the main mode of transmission between dairy cows (Keefe, 2012), a recent study identified the gastrointestinal tract of cows and the surroundings as neglected reservoirs, suggesting an oro-fecal transmission cycle most likely through the drinking water (Jørgensen et al., 2016). Among samples collected contemporaneously from humans and cattle, 54% of the identified MLST sequence types were shared between humans and cattle indicating possible interspecies transmission of GBS strains (Lyhs et al., 2016).

The present study is a follow up on our previous investigation of host preferences among globally disseminated GBS lineages (Sørensen et al., 2010). Here, we compare human and bovine GBS strains obtained from closed environments. We systematically isolated GBS strains from different body sites of dairy cows and herdspersons within the same farms to detect possible transmission between the two hosts. The isolates were characterized biochemically and examined by pulsed-field gel electrophoresis (PFGE). Based on the results, representative GBS strains were genome sequenced and subjected to comprehensive phylogenetic analysis.

The aim of the study was to examine the distribution of GBS in a closed barn environment and to explore whether individual GBS strains are specialists, i.e. adapted to a specific host, or generalists capable of crossing species barriers to colonize or infect different host species (Bäumler and Fang, 2013).

2. Material and methods

2.1. Sample collection

The project was approved by the Danish National Committee on Bioethics (reference no.: 1-10-72-217-15).

Definitions used in this study: A culture means ‘a collection of microbial cells using a nutrition medium’ (Madigan et al., 2019), i.e. successful growth of a nutrition medium inoculated with a specimen. An isolate is a general term for a pure culture of bacteria obtained from a single colony from a primary isolation plate (Tenover et al., 1995); a strain is made up of the descendant of a single isolate in pure culture (Parker and Garrity, 2019); a clone is a population of bacterial cells derived from a single parent cell (Ørskov and Ørskov, 1983).

Samples were collected from lactating cows, employees at dairy herds, and equipment used for milking and milk storage on eight selected farms. The overall herd inclusion criterion was a positive test for GBS in the last annual bulk tank surveillance sample, based on a low cycle threshold value (Ct) in a GBS-specific real-time PCR analysis [Thermo Fisher®, Finland] (Mahmmod et al., 2013). Screening of herds with a Ct value < 30 was repeated shortly before sampling to confirm that the herd was still GBS positive. The owners of GBS positive herds were then contacted, informed, and encouraged to participate in the project. No requests were declined.

In order to examine whether one or more GBS strains were present in the individual herds, we aimed to collect samples at three anatomical sites from at least 25 cows. Based on recent DHI (Dairy Herd Improvement) test results (Schukken et al., 2003) obtained from the Central Husbandry Register (CHR), the cows (29–34 per farm) with the highest somatic cell count at the latest milk recording prior to the herd visit were selected at each farm for sampling. All swab samples were collected by aseptic non-touch technique (ANTT) (Clare and Rowley, 2018) by an experienced veterinarian (MF). Milk samples were taken according to the National Mastitis Council recommendations (NMCRC, 2004) after disinfection of the udder. Three samples, *i.e.* composite quarter foremilk samples (10 ml), and both rectal and vaginal e-swabs (SSI Diagnostica, Hillerød, Denmark) were obtained from each of 239 cows. Before collecting a vaginal swap, the vulva was disinfected and care was taken to sample at least 10 cm into the vagina. The rectal swabs were taken by ANTT, i.e. by sampling from the intestinal wall around 10 cm inside the rectum without touching irrelevant areas. From an additional four cows, the swabs were lost and only milk samples were obtained.

Oropharyngeal and rectal e-swabs were obtained from all herd employees (N = 42), except for two rectal and one oral sample that were missed, and from female employees (N = 8) a vaginal e-swab was obtained as well. All participants gave their informed consent before participation and were instructed how to take the samples. Furthermore, several e-swab samples were collected from various equipment (robot teat brushes, teat cups, milk filter socks, and bulk tank milk; N = 40). Samples were transported to the laboratory on ice and cultured the same day as follows.

One ml of stirred milk samples, and 1 ml of Amies transport medium from e-swab tubes were transferred with sterile plastic pipettes to tubes containing 9 ml of selective enhancement broth (Lim broth, BioMérieux). After overnight incubation at 37 °C, each broth culture was sequentially streaked (4 quadrant method) on selective/indicative CHROMID® Strepto-B agar plates (BioMérieux) by using 10-µL disposable inoculating loops.

Additionally, 100 µL fresh milk samples from randomly selected cows were evenly spread directly (i.e. without enhancement) on Strepto-B agar plates by using disposable Drigalski spatulas. The plates were incubated at 37 °C, examined the following day, and photos were taken for semi-quantitative estimation of bacterial growth.

Plates were incubated overnight (˜ 18 h, 37 °C, 5% CO_2). If culture positive, one or two pale pink to red colonies from each of the Strepto-B plates were picked, transferred, and sequentially streaked on plain 5% blood agar plates (SSI Diagnostica). Plates were incubated overnight at 37 °C in 5% CO_2. Colonies displaying typical growth (smooth, non-pigmented, greyish, slightly convex, 1–2 mm with or without β-hemolysis) were examined by group B strep latex agglutination test (SSI Diagnostica) (Slotved and Hoffmann, 2017) and the isolates were frozen at -80 °C in beef broth with 10% glycerol (SSI Diagnostica). Bacterial identifications were verified by routine MALDI-TOF analysis (mass spectrometry, Microflex LT, Bruker Daltonics) at the Department of Clinical Microbiology, Aarhus University Hospital, Skejby, Denmark.

2.2. Biochemical and antimicrobial susceptibility testing

Fermentation was examined in lactose and salicin medium (SSI Diagnostica). The presence of lactose operons (Lac.1, Lac.2a, Lac2b and/or Lac.2c) were detected by BLAST analyses (see below).

Susceptibility to clindamycin, erythromycin, penicillin G, and tetracycline was tested by a standard disk diffusion method on Müeller Hinton agar plates (Ø 9 cm) supplemented with 5% horse blood (SSI diagnostic) using Neo-Sensitabs tablets (Ø 9 mm) according to the instructions of the manufacturer (Rosco diagnostics, Denmark). After incubation (18 h, 37 °C, 5% CO_2), diameters of inhibition zones around the tablets were measured with a Vernier caliper and sensitivity of each strain was interpreted according to the Rosco user’s guide with reference to the EUCAST Disk Diffusion breakpoints (The European Committee on Antimicrobial Susceptibility Testing; http://www.eucast.org).

2.3. Serotyping and pulsed-field gel electrophoresis

All GBS strains were serotyped (ImmuLex™, SSI) and examined by pulsed-field gel electrophoresis (PFGE) after cutting by the *Sma*I restriction enzyme as previously described (Hansen et al., 2004). Photos of ethidiumbromide-stained PFGE agarose gels were visually inspected and strains were provisionally grouped according to PFGE patterns. To confirm assigned patterns, strains displaying similar DNA fingerprints were reexamined on the same agarose gel in a second PFGE test.

2.4. Genome sequencing

Based on pairwise comparisons of PFGE patterns, serotyping results, and origin, 25 GBS strains were selected for genome sequencing (Table 4). Sequencing by dual-indexed paired-end 2 × 150 bp aiming for 200x of sequencing depth per genome by Illumina NextSeq 500 was performed at the Department of Molecular Medicine, Aarhus University Hospital, Denmark. Briefly, total DNA was prepared from bacterial cultures by using a DNeasy Blood & tissue Kit (Qiagen). Then, 1 ng DNA was used for library preparation using the Nextera XT DNA sample preparation kit in combination with the Nextera XT index Kit (Illumina®) according to the manufacturer's protocol. Paired de-multiplexed fastq files were generated using CASAVA software (Illumina), and initial quality control was performed using the FastQC program http://www.bioinformatics.babraham.ac.uk/projects/fastqc. Reads were assembled using SPAdes Genome Assembler 3.9 (http://bioinf.spbau.ru/en/spades).

2.5. Publicly available GBS genomes

For comparison, publicly available assembled genomes were included in the phylogenetic analyses. We included genomes of strains with origin in Denmark or Italy only, as we had access to genomes of strains from both humans and bovine hosts from these two countries.

A total of 82 public genomes of human (Danish N = 29, Italian, N = 33) and bovine (Danish, 10; Italian, 10) GBS strains were downloaded via the bacterial bioinformatics service (Wattam et al., 2017) at the Pathosystems Resource Integration Center website (PATRIC, University of Chicago, https://www.patricbrc.org). Professor Ruth Zadoks, Institute of Biodiversity Animal Health & Comparative Medicine, University of Glasgow, Glasgow, Scotland kindly provided the information on genomes of 18 European bovine GBS strains (Danish: MRI Z1-012, MRI Z1-022, MRI Z1-023, MRI Z1-025, MRI Z1-035, MRI Z1-039, MRI Z1-048, and MRI Z1-049; isolates obtained through collaboration with Dr. Jørgen Katholm. Italian: MRI Z1-209, MRI Z1-211, MRI Z1-212, MRI Z1-213, MRI Z1-214, MRI Z1-215, MRI Z1-216, MRI Z1-217, MRI Z1-218, and MRI Z1-219; isolates obtained through collaboration with Professor Paolo Moroni. Clinical information and Genbank accession numbers (https://www.ncbi.nlm.nih.gov/genbank/) for the 82 genome-sequenced strains are listed in the online Technical Appendix, Table 2.

By January 2019, 310 human European GBS genomes were available online and they were all included in the BLAST analysis for the presence of the *lacG* gene.

2.6. Phylogenetic analyses

MLST typing was performed on whole genome sequences (Larsen et al., 2012) by uploading the contigs to the online MLST 1.8 service (https://cge.cbs.dtu.dk/services/MLST). For few genomes, the sequence type (ST) was unknown (marked as ST? in Fig. 2).

For SNP (single-nucleotide polymorphism) analysis, public GBS genomes and genomes sequenced in this study were combined in "genome groups" according to the purpose of the individual analyses. The phylogenetic relationships between bacterial isolates were established by SNP based cluster analysis (Kaas et al., 2014). Genomes included in a single analysis (*i.e.* a "genome group") were uploaded collectively to the CSI Phylogeny 1.4 service (https://cge.cbs.dtu.dk/services/CSIPhylogeny) and analyzed by using the default parameters. The genome of a strain randomly selected from each "genome group" was used as internal reference in the analyses. The calculations reveal and include shared and trusted genome positions (Kaas et al., 2014), i.e. the conserved "core genome" in common for the strains included in each analysis. The number of included positions depends on the mutual relationships between the strains in the individual analysis. For each analysis, a minimum evolution tree (the phylogenetic tree with the smallest sum of branch length estimates) was inferred based on the concatenated alignment of the SNPs by using MEGA 7.0.26 (Kumar et al., 2016).

Average Nucleotide Identity (ANI) analysis between genomes was performed by the ANIb method (values are calculated by using BLAST) at the JSpecies website (Richter et al., 2016) [http://jspecies.ribohost.com/jspeciesws/#home]. As an example, the pairwise ANI comparisons, i.e. comparison of all sequences shared between two given strains, were executed on genomes of strains assigned to the first subcluster of the dendrogram shown in Fig. 2. The results were transferred to a spreadsheet (Microsoft Excel®) for calculation of mean values and the generation of a table heat map (Technical appendix Figure 4).

Genome sizes, guanine-cytosine content (GC%), relative core genome size [RCGS (%); core genome size relative to the whole genome size, i.e. core genome plus accessory genes] were calculated by the fragment alignment method using the Gegenees program (Ågren et al., 2012). We used a fragment-size of 200 bp for the "sliding window" and a step-size of 100 bp to obtain a high resolution.

The BLAST application (Basic Local Alignment Search Tool) was accessed at the PATRIC (see above) or the NCBI websites [https://blast.ncbi.nlm.nih.gov/Blast.cgi]. The blastn or the discontiguous megablast function was used for nucleotide-nucleotide searches to find match of query sequence similarity within the individual GBS genomes. *In silico* capsular gene typing was performed by BLAST searches using sequences of published specific primer sets (forward, reverse, and probe) for all serotypes [Ia, Ib, and II – IX] (Breeding et al., 2016) and the sequence of the entire variable region of the capsular locus for the serotypes, except serotype IX (Sheppard et al., 2016). A capsular gene type was assigned to a GBS strain when all tests for that type showed 95% or higher identity over more than 90% of the query sequence length (17) and tests for other gene types were negative (i.e. less than 90%) for that strain. The presence of the lactose operons (Lac.1 or Lac.2 variants) was established by BLAST search using the sequence for each gene of the individual operons (Fig. 3) extracted from representative published genomes: Lac.1, 10 genes (2603 V/R, GenBank: AE009948.1); Lac.2a, 9 genes (FSL S3-026, GenBank: AEXT00000000); Lac.2b, 11 genes (MRI Z1-212, GenBank: ALSC00000000); Lac.2c, 10 genes (DK-NI-014, GenBank: CQEL01000000).

2.7. Data availability

The whole genome-sequences of the 25 *S. agalactiae* strains sequenced in this study are deposited at DDBJ/ENA/GenBank (DNA Data Bank of Japan; European Nucleotide Archive; GenBank, USA: https://www.ncbi.nlm.nih.gov/genbank/) under the accession numbers QKUH00000000 - QKUZ00000000, and QKVA00000000 - QKVF00000000 (Table 4). Data for other genomes included in this study are listed in online Appendix A, supplemental Table 2.

3. Results

At eight dairy farms with a combination of automatic milking systems and parlor milking, 866 swabs and milk samples were collected from 243 cows, 42 herdspersons [34 men and 8 women] (Table 1–3), and from equipment used for milking.

3.1. Bacterial identification and phenotypic characterization

GBS were isolated from 105 of the samples and the identity confirmed by MALDI-TOF. In addition, two isolates initially suspected as GBS (reddish colonies on CHROMID® Strepto-B plates and agglutination in group B latex) were subsequently identified as *Streptococcus uberis* by MALDI-TOF and excluded from the study. GBS were detected by sampling equipment for milk harvesting and milk storage (robot teat brushes, milk filter socks and pooled samples from the bulk milk tanks; 19 samples GBS positive out of 40). Some of the strains obtained from the

Table 1
Prevalence of *Streptococcus agalactiae* in dairy cows and carriage among herdspersons at the eight Danish dairy farms.

	Host	
Farm	Bovine	Human
1	3/30 [a]	1/2
2	2/30	0/9
3	9/34	6/6
4	3/29	3/3
5	21/30	5/9
6	6/30	1/4
7	2/30	3/6
8	4/30	1/3
Total	50/243 [b]	20/42
GBS prevalence/ carriage[c]	21%	48%

[a] Number of GBS carriers / number of examined individuals. For positive individuals, GBS was isolated from one or more sampling sites (Tables 2 and 3).

[b] A total of 243 cows and 42 herdspersons (34 men and 8 women) were examined.

[c] GBS prevalence (percentage of infected - with or without clinical signs –or rectally colonized animals) and GBS carriage rate in herdspersons.

Table 2
Streptococcus agalactiae in samples obtained from dairy cows.

Cow	Bovine samples		
no.:	Milk	Rectal	Vaginal
3B13[a]	–[b]	+	–
3B17	+	+	–
3B28	+	+	–
1B15	+	–	+
3B11	+	–	+
5B21	+	–	+
5B28	+	+	+
N = 43[c]	+	–	–
N = 193[c]	–	–	–
Positive sites[d] (N = 57)	49	4	4

[a] Labeling of animals: Farm no., host (B, bovine), cow no.

[b] –, GBS not detectable; +, GBS isolated from sample.

[c] N: number of animals not singly listed.

[d] Out of 243 examined cows, 50 (21%) were infected or colonized with GBS at one or more sites.

Table 3
Carriage of *Streptococcus agalactiae* by herdspersons at three sampling sites.

	Person	Human samples		
Gender[d]	no.:	Pharynx	Rectal	Vaginal[d]
F	4H3[a]	+	+	–
F	3H1	–	+	+
F	3H2	–	+	+
F	5H4	–	+	+
M	5H2	+	–	
M	5H9	+	–	
M	1H2	–	+	
M	3H3	–	+	
M	3H4	–	+	
M	3H5	–	+	
M	3H6	–	+	
M	4H2	–	+	
M	5H5	–	+	
M	5H6	–	+	
M	6H3	–	+	
M	7H2	–	+	
M	7H6	–	+	
M	4H1	+	+	
M	7H8	+	+	
M	8H3	+	+	
F (N = 4); M (N = 18)	N = 22[c]	–	–	–[e]
	Positive sites[e] (N = 27)	6	18	3

b-, GBS not detectable; +, GBS isolated from sample.

[a] Labeling of individuals: Farm no., host (H, human), subject no.

[c] N: number of individuals not singly listed.

[d] F, female; M, male.

[e] Out of 42 examined employees, 20 (48%) were colonized with GBS at one or more sites.

equipment displayed a PFGE pattern that matched a pattern found among GBS strains obtained from cows at the same farms (ex. online Technical Appendix Fig. 1, lane 15 and 21). Investigation of the surroundings was, however, not performed systematically and is only discussed briefly (data not included). GBS were isolated from one or more sampling sites of 21% (N = 50) of the examined cows (Table 1 and 2), and the GBS prevalence [percentage of infected (with or without clinical signs) and rectally colonized animals] in the livestock at different farms varied from 7% to 30%. The shedding varied among the individual GBS-positive cows as CFU (colony forming units) counts ranged from ˜10^3 to more than 10^6 per mL of milk (online Technical Appendix Fig. 2). GBS were predominantly isolated from udders and most cows were positive at one site only (udder, N = 43 or rectum, N = 1); however, 12% of the GBS-positive cows were colonized or infected at two or three body sites, *i.e.* udder plus rectum and/or vagina (N = 6, Table 2). Surprisingly, 48% of the herdspersons (N = 20) were carriers of GBS at one or more sites (Table 1 and 3), and 14% (N = 6) were colonized in the pharynx. Among the eight female employees, half were colonized at two sites (pharynx and rectum, N = 1; vagina and rectum, N = 3). No correlation between the GBS prevalence in cows and carriage among herdspersons was found by linear regression (data not shown). One isolate from each of the 84 GBS-positive sampling sites (strains obtained from the surroundings were excluded) was examined in detail (N = 57, Table 2 and N = 27, Table 3).

All the isolated GBS strains, except for four of bovine origin, were β-hemolytic and sensitive to penicillin, erythromycin, and clindamycin (one exception, online Technical Appendix Table 3A). Around two-thirds of the strains fermented salicin (53/84) with no difference between strains obtained from humans and cattle. All the 57 examined bovine strains and 43% (12/27) of the human strains fermented lactose.

BLAST searches showed that 104 out of the 107 GBS strains included in the phylogenetic analyses (see below) possessed a complete lactose operon designated Lac.1 (Richards et al., 2011). Additionally, all 33 bovine strains and 15 out of 74 human strains possessed a single or two lactose operons designated Lac.2 (suppl. Table 2). Three Lac.2 variants were revealed among the examined strains. We designated theses variants Lac.2a, b and c (Fig. 3). BLAST analyses showed only limited nucleotide sequence similarity between orthologous genes from the three variants (selected examples in Fig. 3), which indicates independent evolution and sequence diversification for each variant of the Lac.2 operon. In contrast to the Lac.1 operon, all the three Lac.2 variants possesses the gene *lacG*, which encodes the enzyme β-galactosidase (Fig. 3). The BLAST analyses demonstrated that *lacG*, was always a part of the Lac.2 operon (suppl. Table 2) - a prerequisite for lactose fermentation (Iskandar et al., 2019). Additional BLAST search revealed the presence of *lacG* in 31 out of 310 public human European GBS genomes obtained from individuals without apparent relation to farming. Thus, around 10% of the human GBS reservoir in Europe possess the β-galactosidase gene/ the Lac.2 operon. i.e. lactose fermentation is not restricted to bovine strains.

More bovine (29/57, 51%) than human strains (3/27, 11%) were resistant to tetracycline. However, 23 out of the 29 resistant bovine isolates and the three resistant human isolates were collected at Farm 5, indicating local dissemination of a resistant clone.

The prevalence of various serotypes differed between the farms. Thirteen serotype III strains (11 bovine and 2 human) were detected at Farm 3, 24 serotype V strains (21 bovine and 3 human) were detected at

Table 4
Biochemical and serological characteristics of 25 *Streptococcus agalactiae* strains selected for whole genome sequencing.

Position [a]	Strain	Lactose	Farm	Host	Serotype	ST [b]	Accession no. [c]
21	1B13 M [d]	+ [e]	1	B [f]	V [g]	1	QKVF00000000
60	3B11V	+	3	B	III	23	QKVE00000000
61	3B13R	+	3	B	III	23	QKVD00000000
62	3B21M	+	3	B	III	23	QKVC00000000
73	3H1V	–	3	H	Ia	23	QKVB00000000
63	3H3R	+	3	H	III	23	QKVA00000000
79	4B14M	+	4	B	IX	130	QKUZ00000000
82	4H1O	+	4	H	IX	130	QKUY00000000
81	4H2R	–	4	H	IX	130	QKUX00000000
80	4H3O	+	4	H	IX	130	QKUW00000000
14	5B15M	+	5	B	V	1	QKUV00000000
11	5B20M	+	5	B	V	1	QKUU00000000
16	5B21V	+	5	B	NT/V	1	QKUT00000000
15	5B28V	+	5	B	V	1	QKUS00000000
13	5B2M	+	5	B	V	1	QKUR00000000
18	5H2O	+	5	H	V	1	QKUQ00000000
44	5H4R	–	5	H	Ia	?	QKUP00000000
12	5H5R	+	5	H	V	1	QKUO00000000
71	5H6R	–	5	H	Ia	23	QKUN00000000
19	5H9O	+	5	H	V	1	QKUM00000000
17	6B19M	+	6	B	V	1	QKUL00000000
106	7B18M	+	7	B	Ia	?	QKUK00000000
107	7H8O	+	7	H	Ia	314	QKUJ00000000
105	8B14M	+	8	B	Ia	103	QKUI00000000
52	8H3O	+	8	H	Ia	110	QKUH00000000

[a] Position in the phylogenetic tree Fig. 2.
[b] ST: MLST sequence type types.?, unknown ST.
[c] DDBJ/ENA/GenBank accession numbers (see text).
[d] Labeling of GBS strains: Farm no., host (B, bovine; H, human), subject no., sampling site (M, milk; O, oropharynx; R, rectal; V, vaginal).
[e] Lactose fermentation: +, positive; -, negative.
[f] Host: B, bovine; H, human.
[g] Serotype: typed by latex agglutination and confirmed by capsular gene typing. NT/V, non-typeable by latex, serotype V in capsular gene typing.

Farm 5, and eight serotype IX strains were detected at Farm 4 (3 bovine and 5 human) (Online Technical Appendix Table 1B and C).

3.2. Phylogenetic analysis of GBS strains

PFGE analyses revealed 24 patterns among the 84 strains. Some were unique to a single strain while others were shared by several strains obtained from different cows, herdspersons, and equipment (ex. online Technical Appendix Fig. 1). To pursue the apparent relationships among some of the strains obtained from the two different hosts, 25 of the 84 isolates were selected for detailed phylogenetic analysis based on whole genome sequences (Table 4). The genomes consisted of 2.1–2.2 × 10^6 bp (base pairs) with a GC content of 34.95–35.80%.

The minimum-evolution tree generated from variable sites (N = 19,154) of shared sequences (SNPs) shows that cattle and humans within the individual farms often share the same clone of GBS (Fig. 1; farm 3, 4, 5, and 7). This result demonstrates lack of host specificity for some lineage of group B streptococci.

A bovine and a human strain collected at farm 8 (strains 8H3O and 8B14 M) were unrelated. Three other human strains (3H1V, 5H6R, and 5H4R) did not cluster with bovine strains from the same farms in the primary analysis. In contrast to the other strains, the three latter human isolates were all lactose negative (Table 4) and as expected their genomes did not contain the Lac.2 operon (suppl. Table 2).

In order to evaluate the findings in a broader context, the 25 sequenced genomes were compared with publicly available genome sequences for an additional 82 GBS strains. Bovine and human strains from two European countries (Denmark and Italy) were included (appendix A Table 2). As above, the relationship between all the 107 strains was inferred by a SNP analysis followed by construction of a minimum-evolution tree [variable sites, N = 24,773; the proportion of positions shared and trusted by each isolate and the reference genome was between 81.4 and 98.3% (average 89.5%); the proportion of the reference genome shared by all 107 isolates was 62.8%]. The dendrogram displayed a number of sub-clusters most of which consisted of both bovine and human strains (e.g. positions 1–21, 23–34, 37–48, 49–59, 60–66, 78–82 and 104–107; Fig. 2). The 25 strains from the current study were located in different parts of the phylogenetic tree and they clustered neither according to host nor according to country of origin. One sub-cluster consisted of human strains only (mainly ST17 strains, position 86–103, Fig. 2); but none of the GBS strains collected during the current study were assigned to that part of the tree.

As an example, the clonal relationship between bovine and human GBS strains assigned to the same sub-cluster by the SPN analysis (positions 1–22; Fig. 2) was examined further by pairwise ANI comparisons (online Technical appendix A Figure 4). All ANI-values for this sub-cluster were above 99.35% although the strains were obtained from two different countries and, in some instances, from humans without relation to farming. The percentage of the query's genome conserved in the reference genome was above 92.5 for all the pairwise comparison of strains assigned to this sub-cluster. The bovine (positions 11, 13–16; Fig. 2) and human (positions 12, 18 and 19) strains collected at farm 5 shared more than 98.5% of their genomes. Pairwise comparisons of the relative core genome sizes (RCGS) definitely confirmed the clonal relationship between bovine and human strains in this sub-cluster. For all genome pairs the RCGS values were higher than 92% despite that the strains were obtained from two different hosts in two different countries (Denmark and Italy). For bovine and human GBS strains collected at Farm 5 the RCGS values were close to 100% (Suppl. Fig. 4), i.e. the strains had almost identical genomes.

Among the 107 included genomes, the two most distantly related were from the bovine strains 1B13 M (position 21, Fig. 2) and 8B14 M (position 105) both collected in this study. The ANI-value for this pair of GBS strains was 97.17 and RCGS was 88.4%.

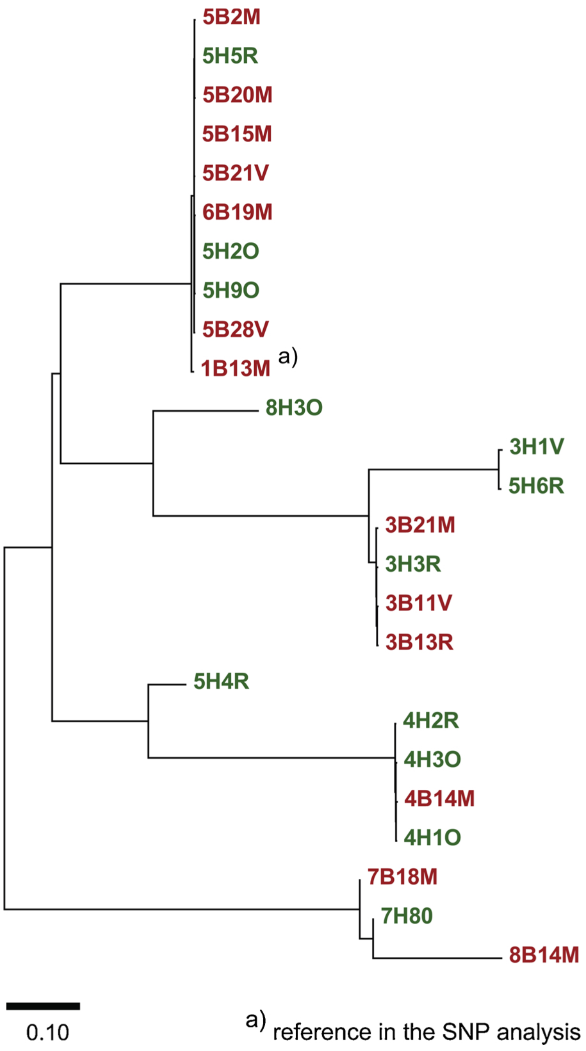
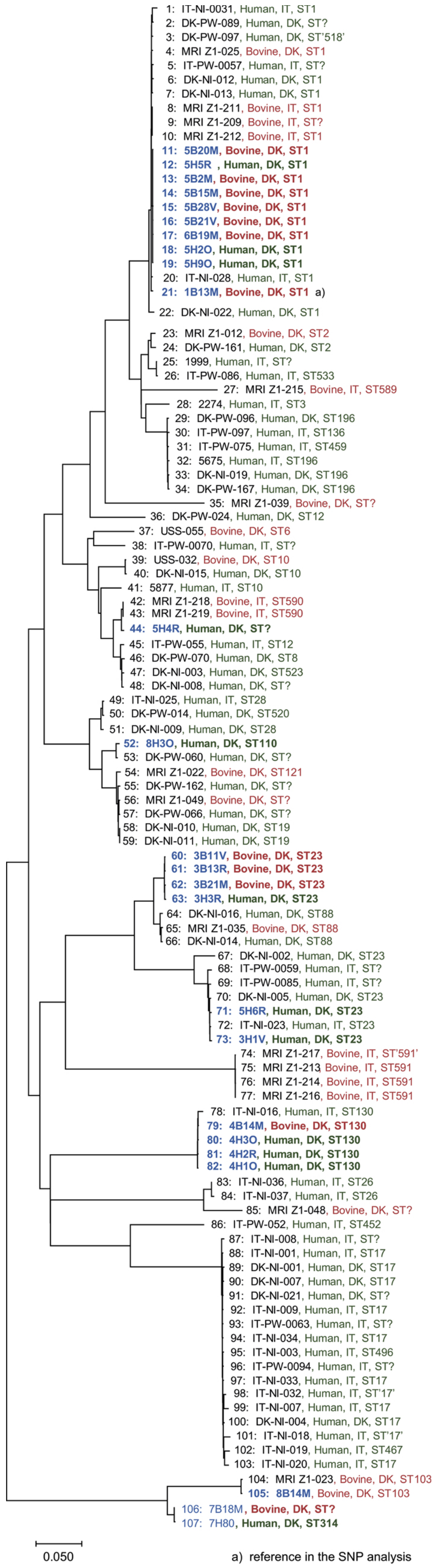


Fig. 1. Phylogenetic relationship between 12 human (green) and 13 bovine (red) *Streptococcus agalactiae* (GBS) strains obtained from 7 Danish farms. The dendrogram is based on variable sites in whole genome-sequences (see text for details). The figure shows that GBS strains obtained from the human and bovine host at a farm belong to the same clone. Thus, the relationships do not depend on the host species but on the place of origin. The evolutionary distances are in the units of the number of base substitutions per site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

Only few other studies (Manning et al., 2010) were designed to compare GBS strains obtained from cows and herdspersons at the same farms in order to explore the host specificity for this pathogen. Our investigations identified several distinct GBS clones that were carried both by animals and herdspersons at the same farms. As shown by the detailed phylogenetic analysis, the isolates clustered neither according to country of origin nor host species. In contrast, clones shared by the two host species were located in different sub-clusters together with other human strains obtained from persons with no relation to farming This suggests a human reservoir of clones capable of colonizing both cattle and humans.

One of our previous investigations revealed on the one hand global evolutionary GBS lineages apparently adapted to a specific host species (e.g. clonal complex CC17 in the human host; CC67 in the bovine host) and on the other hand “a core population” of GBS, that lacks tropism for a specific host. However, apparent association between GBS lineages and different hosts may also reflect physical or other transmission barriers between populations than host tropism, which may cause biased sampling (Sørensen et al., 2010).

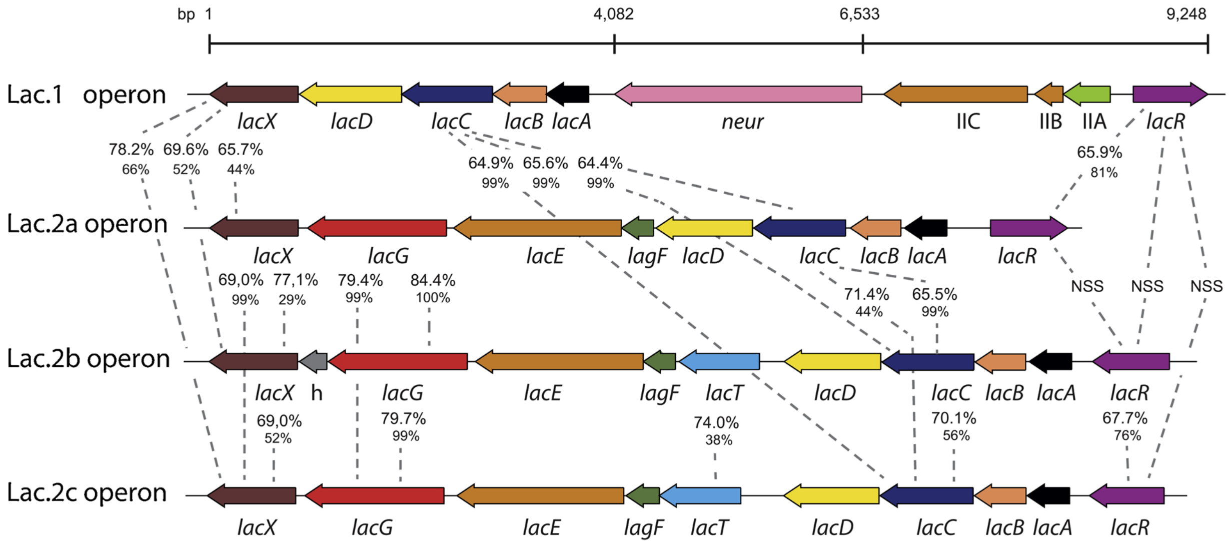


(caption on next page)

Fig. 2. Phylogenetic relationship between human and bovine *Streptococcus agalactiae* (GBS) strains illustrated by a dendrogram based on SNP-based analysis of whole genome sequences. The analysis included human (N = 74) and bovine (N = 33) strains from Denmark and Italy (see Table 4 and online Technical Appendix Table 2). Each label in the figure indicates a genome [position in the tree and strain designation; public genomes, black font; genome sequenced in the current study, blue bold font; host (human, green; bovine, red); country (DK, Denmark; IT, Italy) and ST (MLST sequence type)]. Most sub-clusters consist of strains obtained from both the human and the bovine host. The evolutionary distances are in the units of the number of base substitutions per site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

GBS co-colonization of human couples has been reported (Foxman et al., 2006; Manning et al., 2000, 2004; Manning et al., 2002). Inter-species transmission of GBS from the human reservoir to dairy cows has also been suggested in the past but experimental evidence was limited (Jensen, 1985; Lyhs et al., 2016; Manning et al., 2010; Oliveira et al., 2006). Our finding of GBS clones shared by humans and cattle at 4 out of 8 farms (Fig. 2) support that direct transmission of GBS between different host species is frequent when the two hosts occupy the same environment demonstrating lack of host specificity in at least part of the GBS population.

GBS utilize lactose by the tagatose-6 phosphate pathway (Iskandar et al., 2019) regulated by the Lac.2 operon (Richards et al., 2011). Richards et al. suggested that bovine- and human-adapted GBS strains have distinct lactose utilization pathways. *LacG* encoding β-galactosidase is missing in the Lac.1 operon, which is not involved in lactose fermentation. Instead, Lac.1 was suggested to have a function in relation to the neuraminidase-like enzyme (a possible virulence factor) and to be specific for human strains. In contrast, Lac.2 was reported to regulate lactose utilization specifically in bovine strains (Richards et al., 2011). In the genome, the lactose operons are flanked by integrases, and like other integrative and conjugative elements (Ambroset et al., 2015), they may be transferred between strains by lateral gene transfer through the GBS population. In agreement with this perception, we found the presence of the Lac.2 operon in unrelated phylogenetic lineages and sub-clusters (suppl. Table 2). Therefore, our findings do not support the concept that bovine and human GBS strains have distinct lactose utilization pathways. However, in agreement with the



Gene no.	Gene	Function	Lac.1 operon (2603V/R)
1	*lacX*	🟫	Aldolase 1 epimerase LacX
2	*lacD*	🟨	Tagatose 1,6-bisphosphate aldolase
3	*lacC*	🟦	Tagatose-6-phosphate kinase
4	*lacB*	🟧	Galactose-6-phosphate isomerase, LacB subunit
5	*lacA*	⬛	Galactose-6-phosphate isomerase, LacA subunit
6	neur	🟪	Sialidase / neuraminidase-like protein
7	IIC	🟫	PTS system, galactose-specific IIC component
8	IIB	🟫	PTS system, galactose-specific IIB component
9	IIA	🟩	PTS system, galactose-specific IIA component
10	*lacR*	🟪	Lactose phosphotransferase system repressor

Gene no.	Gene	Function	Lac.2a operon (FSL S3-026)
1	*lacX*	🟫	Aldolase 1 epimerase LacX
2	*lacG*	🟥	6-phospho-beta-galactosidase
3	*lacE*	🟫	PTS system, lactose-specific IIB /IIC component
4	*lacF*	🟩	PTS system, lactose-specific IIA component
5	*lacD*	🟨	Tagatose 1,6-bisphosphate aldolase
6	*lacC*	🟦	Tagatose-6-phosphate kinase
7	*lacB*	🟧	Galactose-6-phosphate isomerase, LacB subunit
8	*lacA*	⬛	Galactose-6-phosphate isomerase, LacA subunit
9	*lacR*	🟪	Lactose phosphotransferase system repressor

Gene no.	Gene	Function	Lac.2b operon (MRI Z1-212)
1	*lacX*	🟫	Aldolase 1 epimerase LacX
2	h	⬜	Hypothetical protein
3	*lacG*	🟥	6-phospho-beta-galactosidase
4	*lacE*	🟫	PTS system, lactose-specific IIB /IIC component
5	*lacF*	🟩	PTS system, lactose-specific IIA component
6	*lacT*	🟦	Beta-glucoside bgl operon antiterminator
7	*lacD*	🟨	Tagatose 1,6-bisphosphate aldolase
8	*lacC*	🟦	Tagatose-6-phosphate kinase
9	*lacB*	🟧	Galactose-6-phosphate isomerase, LacB subunit
10	*lacA*	⬛	Galactose-6-phosphate isomerase, LacA subunit
11	*lacR*	🟪	Lactose phosphotransferase system repressor

Gene no.	Gene	Function	Lac.2c operon (DK-NI-014)
1	*lacX*	🟫	Aldolase 1 epimerase LacX
2	*lacG*	🟥	6-phospho-beta-galactosidase
3	*lacE*	🟫	PTS system, lactose-specific IIB /IIC component
4	*lacF*	🟩	PTS system, lactose-specific IIA component
5	*lacT*	🟦	Beta-glucoside bgl operon antiterminator
6	*lacD*	🟨	Tagatose 1,6-bisphosphate aldolase
7	*lacC*	🟦	Tagatose-6-phosphate kinase
8	*lacB*	🟧	Galactose-6-phosphate isomerase, LacB subunit
9	*lacA*	⬛	Galactose-6-phosphate isomerase, LacA subunit
10	*lacR*	🟪	Lactose phosphotransferase system repressor

xx% - - - - Percent nucleotide identity (blastn)
yy% Query cover (blastn) between homologous genes placed above and below the the stippled line (selected examples), NSS: No significant similarity

Fig. 3. Diagrammatic representation of the gene organization within lactose operons Lac.1 and Lac.2 (Richards et al., 2011) of *Streptococcus agalactiae*. Three variants of the Lac.2 operon were revealed among the 107 examined GBS genomes and designated 2a, 2b, and 2c. Figures and stippled lines indicate percent nucleotide identity / query cover between homologous genes as revealed by BLAST analyses. Colored boxes indicate functional identity as revealed by the annotation (Iskandar et al., 2019).

previous study (Richards et al., 2011), our results support that lactose fermenting GBS strains possess a selective advantage in the ability to colonize and infect cattle compared to strains that do not utilize this disaccharide.

As reported by others (de Aguiar et al., 2016; Jørgensen et al., 2016), the present study demonstrates a broad ecological reservoir for GBS. Thus, colonization was detected in the oropharynx, intestine, and vagina of humans and in the udder, intestine, and vagina of dairy cows. Additionally, GBS were isolated from various equipment at the farms.

In herds with robotic milking systems, humans have less contact with the animals in comparison with farmers who use a conventional milking procedure. However, contact does take place during calving, milking of colostrum, and training of the cow to the robot. Perhaps more importantly, interviews of employees participating in our study showed that all of them now and then drink fresh untreated (i.e. unpasteurized) and sometimes GBS-contaminated milk at the farms, an observation that may explain the oropharyngeal colonization in some persons.

In a previous investigation (Katholm and Rattenborg, 2009), it was found that 45% (N = 105) of 233 Danish GBS infected dairy herds had cattle introduced from other infected farms. Another 45% (N = 104) of the herds had only received cattle from GBS-free herds and 10% (N = 24) of the infected herds had not introduced animals from other farms at all. The latest information from the Danish Central Husbandry Register shows the same trend. In 2015, 13 farms changed status from GBS-free to newly GBS-infected and 10 of these farms had no previous records of GBS-infected animals for more than three years. It was suggested (Katholm and Rattenborg, 2009) that humans most likely transmitted the GBS to the cattle in the latter situations. This conclusion is in agreement with our results. GBS strains isolated from Danish dairy cattle in the present study belonged to the same sub-clusters as clones commonly circulating in human population in two European countries. On one side, these observations indicate that humans may initially introduce GBS to an infection-free herd and that the contagion then disseminates between both cows and herdspersons within the individual farms. On the other hand, our results indicate that consumptions of untreated milk may transmit GBS from cattle to humans. Apparently, GBS is the cause of both zoonosis and anthroponosis. Thus, in the interface between animals, humans, and the barn environment, several modes of GBS transmission are possible. In a previous study, we showed some human GBS carriers persistently colonized by a single GBS clone for up to 2 years (Hansen et al., 2004). It would be relevant to examine whether the GBS colonization of herdspersons is permanent or transient.

In conclusion, European GBS strains do not cluster according to country of origin or host, and humans and cattle often share the same GBS clone, which should be taken into consideration in mastitis prevention plans. Thus, our study supports the recommendations for control of *S. agalactiae* in dairy herds and especially for prevention of transmission of the bacteria between herdspersons and cattle by improving hygiene (Jørgensen et al., 2016) since GBS do not exhibit host specificity.

Funding

This study was supported by grant from the Danish foundations “Promilleafgiftsfonden for Landbrug” (the per mille levy foundation for agriculture) and “Frøknerne Anna og Dagny Hjerrilds Fond” (Mss. Anna and Dagny Hjerrild’s Foundation).

Acknowledgement

We thank Ruth Zadoks for providing information on the European bovine *Streptococcus agalactiae* strains. We also thank Niels Nørskov Lauritsen for his help in performing the Illumina sequencing of our strains, Trine Schousen Kristiansen and Katrine Dalby Nielsen for their assistance in collecting the samples, and Herdis Berg Johansen for her skillful work in the laboratory.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.06.008.

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