STREPTOCOCCUS AGALACTIAE: A POSSIBLE EXPLANATION FOR RE-EMERGENCE ON DAIRY FARMS IN DENMARK

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Introduction

Streptoccus agalactiae is a gram positive bacterium and is also known in medical literature as group B Streptococcus (GBS). *S. agalactiae* has been isolated from humans and a wide range of animals including humans, fish, sea mammals and cattle (Jensen and Aarestrup 1996, Mian et al. 2009, Pollock 2010). In humans it is a commensal of the throat, gastrointestinal and genitourinary tract (Mhalu 1976) and the prevalence is high (Jensen 1980). *S. agalactiae* is also of clinical importance in pregnant women, as it can cause still-birth or fetal-pneumonia, -meningitis and -sepsis (Baker et al. 2000). In fish the bacterium causes sepsis and meningo-encephalitis (Dellanoy 2011*). S. agalactiae* has been found in stranded sea mammals from Scotland. It has been suggested that infection with *S. agalactiae* may have weakened the animals which may have resulted in death by other means (Pollock 2010).Presence of *S. agalactiae* in sea mammals, which are not considered to be natural hosts of the organism, may be an indicator of faecal pollution of coastal waters. In cattle the bacterium causes sub clinical and clinical mastitis. In the past *S. agalactiae* was one of the most important causes of mastitis in several countries including the USA, Canada, The Netherlands and Denmark (Jensen 1980, Keefe 1994, Loeffler et al. 1995).

Mastitis is inflammation of the mammary gland. In cattle, mastitis is mostly due to infection of the udder by bacteria. It leads to high losses on dairy farms due to a decline in milk yield, veterinary costs and penalties for poor quality milk (Katholm 2010, Keefe 1997). Important bacteria which can cause mastitis on dairy farms in the Netherlands and the UK are: *Streptococcus uberis, Escherichia coli, Staphylococcus aureus, Corynebacterium* species and *Streptococcus dysgalactiae* (Bradley et al. 2007, Loeffler et al. 1995). S. *S. agalactiae* is not one of the main bacteria which causes mastitis anymore in these countries because of strict eradication programs which contain hygiene measures and use of penicillin (Keefe 1997, Loeffler et al. 1995). Hygiene measures are: improving methods of mechanical milking, optimal adjustment of the milking machine, teat-dipping or spraying with a suitable disinfectans after milking, treating dry cows with antimicrobials, correct treatment of clinical mastitis and culling of cows with chronic mastitis (Loeffler et al. 1995).

In Denmark *S. agalactiae* has been systematically controlled for many years. Bulk tank sampling showed a decreased herd prevalence of 20-30 % in the 1950's to a stable 1-2% from 1979 to 2000. Although control programs have not changed dramatically an increase in prevalence is seen, up to 8.2 % in 2009 (Jensen 1980, Katholm 2010). Possible explanations for this increase are: changes in the bacterium itself, e.g. changed virulence; changes in the environment, e.g. changes in hygiene- and management program; or changes in the host animal, e.g. changes in the immune-system, genetics, production level or energy balance.

Using molecular typing methods like serotyping and MLST (for more information about these methods see subsection about molecular typing) human strains and bovine strains are shown to be distinctive (Bisharat et al. 2004). Seventy-six percent of bovine strains could not be serotyped when 96.7% of the human strains were serotyped (Bisharat et al. 2004). In bovine isolates sequence type 67 (ST-67) was most commonly found, whilst ST-67 was not found in human isolates. In human isolates ST-17, followed by ST-1 were most commonly found and these sequence types were not found in any of the bovine isolates. ST-19 and ST-23 were found mostly in human isolates but were also found in a few bovine isolates (Bisharat et al. 2004). In an experimental challenge study Jensen shows that in cattle human strains of *S. agalactiae* can cause mastitis with a more acute character than bovine strains. Cows infected with human strains show a higher self-cure rate than cows infected by bovine strains. It was also thought that human strains do not easily spread from cow to cow (Jensen 1982).

Bacterial molecular typing is discriminating between isolates of bacterial species. In the case of genotyping, discriminations are made based on DNA and can be categorized as single locus or multiple loci approaches (Spratt 1999). Multi Locus Sequence Typing (MLST) was introduced in the late 1990's and has been a helpful tool in molecular epidemiology of bacterial diseases. Many MLST schemes use 450-500 base pair fragments of seven housekeeping genes. These fragments are amplified using Polymerase Chain Reaction (PCR) and amplicons are then sequenced. Sequences for each of the seven fragments are assigned an allele and an allelic profile or sequence type (ST) is then assigned to each unique combination of the alleles (Enright and Spratt 1999, Jones et al. 2003, Spratt 1999). An eBURST diagram can be made subdividing MLST data sets into non overlapping groups of related STs or clonal complexes (Feil et al. 2004) (fig.1).

In our study we investigated if *S. agalactiae* isolates from different countries (Denmark and Scotland) different host species (seals and cattle) and different years. Both the geographic distance and long term epidemiological questions about origin require a molecular typing method that uses genes that are highly conserved and won't change quickly. MLST uses housekeeping genes which encode essential cell functions. Any change in these genes could be disadvantageous to the bacterium. MLST of *S. agalactiae* is used in many different studies, host species and syndromes. All results obtained in these studies are available online (www.mlst.net) (Jones et al. 2003, Spratt 1999). MLST is the typing method of choice for our study because it covers the appropriate spatiotemporal scale and because it allows for comparison with data from other studies.

In our study we also used 9 'alternative' housekeeping genes that are present throughout the *S. agalactiae* genome (fig. 2.) In order to get more information whether the isolates we used grouped with human- or bovine strains and also because capsular polysaccharides have been found to be an important virulence factor we also used capsular serotyping. Capsular serotyping is a typing method that discriminates between bacteria based on their capsular polysaccharides, which have been found to be an important virulence factor. This method has been used for a longer period of time to type *S. agalactiae* and nine different serotypes are recognized (Cieslewicz et al. 2005). Their distribution varies according to geographic location, host species and sequence type (Bisharat et al. 2004, Lachenauer et al. 1999). Sorensen found that most human ST-23 isolates had capsular type Ia and most bovine ST-23 isolates had capsular type III (Sorensen et al. 2010). Another difference between human- and bovine strains is that in the past serotyping has sometimes not worked well for bovine isolates (Dogan et al. 2005). The alternative housekeeping genes and the serotyping were used to determine whether ST23 isolates from seals and from bulk tank milk belonged to human-associated or bovine-associated subtypes, because this would give an indication about the source of infection for seals and cattle.

A helpful tool to get more insight about virulence factors and niche adaptation is virulence genes screening. Richards showed that specific virulence genes (fig. 3.) are more commonly or exclusively found in bovine strains compared to human strains (Richards et al. 2012). He also found that when different Streptococcus species shared the same environment they could exchange genes through 'Lateral Gene Transfer' LGT. We hypothesized that acquisition of virulence genes by human-derived strains of *S. agalactiae* might give them a survival advantage in cattle. An example is the lac operon, which codes for lactose fermentation and may be essential for survival in the bovine udder(Richards et al.). To further investigate how essential the lac operon is to grow in milk, we grew all isolates in commercially available milk (appendix 6).

Using molecular typing methods describe above we tried to find (i) a possible source for *S. agalactiae* in sea mammals and (ii) an explanation for the re-emergence of *S. agalactiae* on Danish dairy farms. The use of isolates from multiple host species and countries in this study was thought to increase the chances to see variety between alleles obtained with MLST. Because of previous studies (Yildirim et al. 2002, Pollock 2010) we expect that the canine- and sea mammal isolates originate from human strains. Preliminary data from Denmark showed that bovine isolates may also belong to human strains, although it is not known why human strains would have emerged in dairy cattle (Zadoks et al. 2011). Screening for virulence genes we expect that isolates coming from bulk tank milk from dairy farms in Denmark will at least contain the lac operon and possibly also other virulence genes, which may have been acquired through lateral gene transfer (LGT) and enhance their survival and spread in cattle herds.

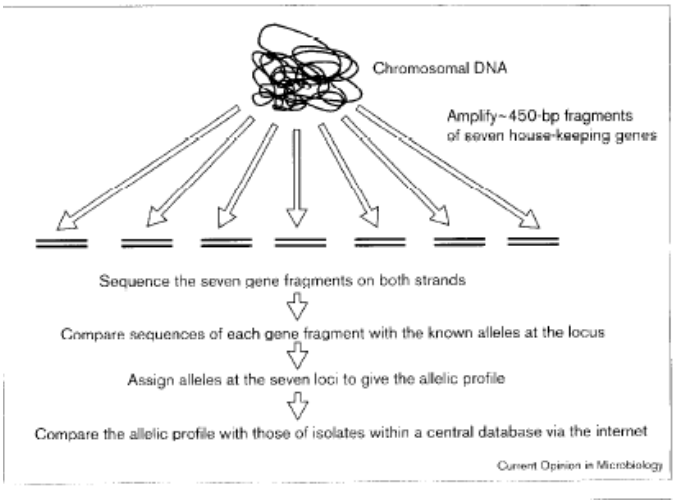

Figure 1. MLST process (Spratt 1999)

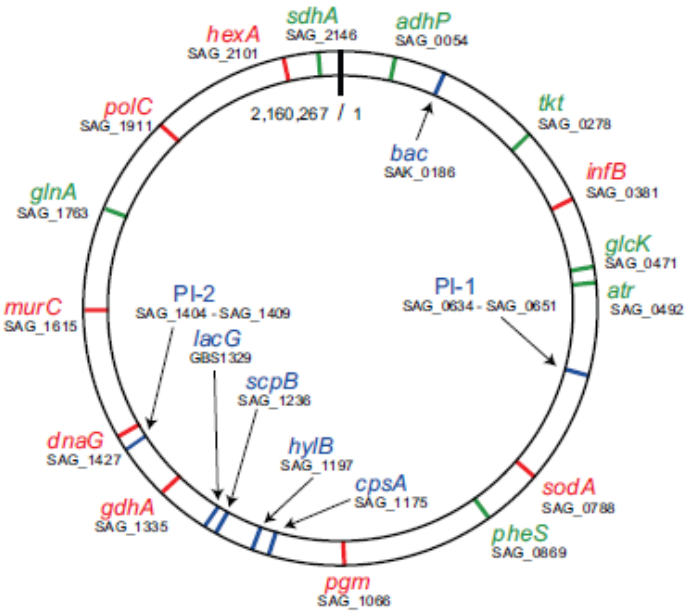

Fig 2. Schematic map of the *S. agalactiae* genome. Genes used in conventional MLST are shown in green. Eight genes we used for MLST are shown in red. We also used scpB shown in blue (fig. 2. from 'Emergence and Global Dissemination of Host-Specific *Streptococcus agalactiae* Clones' 2010 by Sørensen et al.

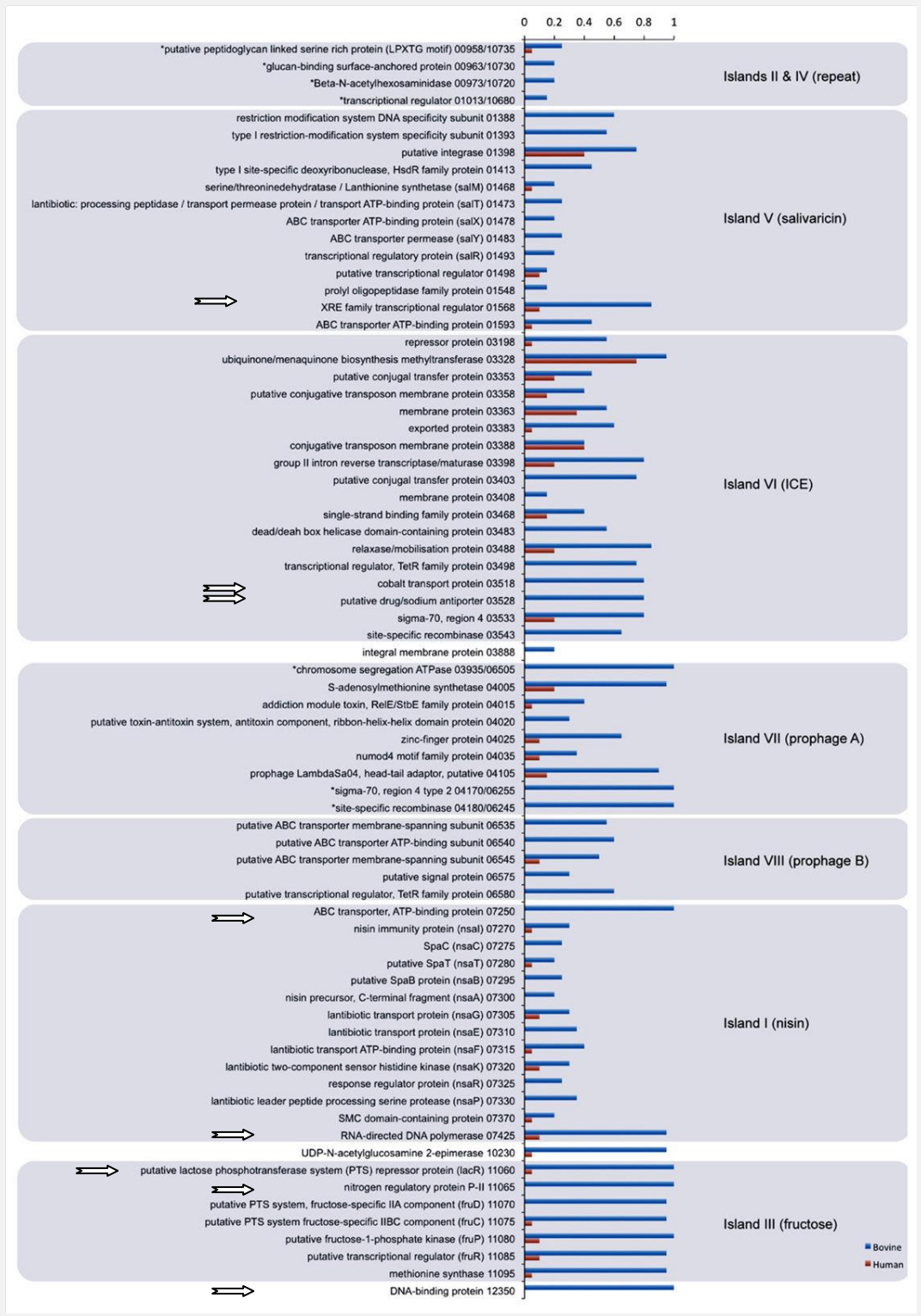

Fig. 3. Results of Richards PCR screening for presence/absence of 73 orthologs identified as specific to *Streptococcus agalactiae* bovine strain FSL S3-026 when compared to eight human GBS strains; 20 bovine and human GBS strains were screened (bovine strains included FSL S3-026 as a positive control). Blue bars show frequency of occurrence in bovine GBS strains and red bars show frequency for human GBS strains. Arrows showing genes we used. (fig. 3. From 'Comparitive genomics and the role of lateral gene transfer in the evolution of bovine adapted *Streptococcus agalactiae*' 2011 by Richards et al)

Material and Methods

Isolates

We used isolates from sea mammals and a dog from Scotland and isolates from dairy cattle from Denmark. Isolates from Denmark all came from different farms to avoid using one particular strain more than once (table 1.).

MRI no.	Original no./herd no	Origin	Source	ST	Application
MRI Z1-197	M215/90/1	Sea mammal	SAC Inverness UK	ST23	MLST
MRI Z1-199	M451/02/4	Sea mammal	SAC Inverness UK	ST23	MLST
MRI Z1-200	M559/02/2	Sea mammal	SAC Inverness UK	ST23	MLST
MRI Z1-201	M59/03/1	Sea mammal	SAC Inverness UK	ST23	MLST
MRI Z1-202	M93/03/2	Sea mammal	SAC Inverness UK	ST23	MLST
MRI Z1-203	M4/06/2	Sea mammal	SAC Inverness UK	ST23	MLST
MRI Z1-204	M2743/98/1	Canine	SAC Inverness UK	ST23	MLST
MRI Z1-208	M302/10/1	Sea Mammal	SAC Inverness UK	unknown	MLST, CPS
MRI Z1-595	M31/11/2	Seal	SAC Inverness UK	unknown	MLST, CPS
MRI Z1-709	M280/11	Grey Seal Pup	Middlebank wildlife centre UK	unknown	MLST, CPS
MRI Z1-024	1	Bovine	Denmark	ST23	MLST, CPS, VGS
MRI Z1-060	110	Bovine	Denmark	ST23	MLST, CPS, VGS
MRI Z1-071	121	Bovine	Denmark	ST23	MLST, CPS, VGS
MRI Z1-123	163	Bovine	Denmark	ST23	MLST, CPS, VGS
MRI Z1-123	73	Bovine	Denmark	ST23	MLST, CPS
MRI Z1-165	178	Bovine	Denmark	ST23	VGS
MRI Z1-025		Bovine	Denmark	ST1	VGS
MRI Z1-054	104	Bovine	Denmark	ST1	VGS
MRI Z1-073		Bovine	Denmark	ST1	VGS
MRI Z1-101	51	Bovine	Denmark	ST1	VGS
MRI Z1-180	187	Bovine	Denmark	ST1	VGS
MRI Z1-023		Bovine	Denmark	ST103	VGS
MRI Z1-084		Bovine	Denmark	ST103	VGS
MRI Z1-087	136	Bovine	Denmark	ST103	VGS
MRI Z1-169	190	Bovine	Denmark	ST103	VGS
MRI Z1-226	124	Bovine	Denmark	ST103	VGS

Table 1. Isolates used for molecular epidemiology investigations. SAC Inverness, Scottish Agricultural College of Inverness UK. MLST, Multi Locus Sequence Typing. CPS, Capsular Serotyping. VGS, Virulence Gene Serotyping.

Bacterial cultures

Before running a PCR we made bacterial cultures of each isolate in order to see what the colonies looked like and if they were a pure culture (appendix 1.).

Extraction of DNA

To extract DNA from bacterial cultures made previously we used the Boiling Method for extraction of DNA (appendix 2.).

Species confirmation PCR

Each time after preparing bacterial cultures and DNA extraction we tested bacterial DNA with *S. agalactiae* specific primers to ensure DNA came from an *S. agalactiae* isolate.
To prepare the PCR mix, 12.5 µl Go Taq Green Master Mix (Sigma, Dorset, United Kingdom), 2.5 µl Forward- and 2.5 µl Reverse primers and 5.5 µl nuclease free water were added. We added 2 µl of DNA (earlier obtained) from each isolate to 23 µl of the PCR mix. Primers RZsag-F and RZsag-R (Phuektes et al. 2001) were used to target 16s to 23s rRNA spacer regions in the bacterial genome (table 2.). PCR products were run on a 1.5% agarose gel in 1x Tris-acetate-EDTA (TAE) buffer (Sigma, Dorset, UK), with gel red (7.5 µl in 100 ml) for 60 minutes with a 100bp marker (Consort EV243 Electrophoresis Power Supply: 100V, 300mA, 50W).

Nine housekeeping genes MLST Gradient PCR

In order to specify PCR conditions for all nine housekeeping genes MLST primers we ran a gradient PCR with one isolate (MRI Z1-202) and 3 primersets (soda, dnaG and hexA) (table 2.). These 3 primers sets were chosen because they had either the highest annealing temperature or the lowest annealing temperature according to information about the annealing temperature given by primers supplier Eurofins (Ebersberd, Germany), or an annealing temperature in between the two other primers. As the temperature for annealing increases the specificity for the primers increases but the sensitivity decreases. We tried to find an annealing temperature that was low enough to be working for all primers, but high enough to be specific for the target region of the bacterial DNA. The gradient for the annealing step was set to go from 50.2°C to 70.4°C for 45 seconds. This temperature range was based on information about the annealing temperature given by primers supplier Eurofins (table 2. for PCR conditions). PCR products were run on a 1.5% agarose gel as described for confirmation PCR.

Table 2. Primers and PCR Conditions

Nine housekeeping genes MLST

We used 10 isolates from Scotland coming from 9 sea mammals and 1 dog. Three of the sea mammal isolates were thought (but to be confirmed) to be ST-23, all other isolates were confirmed to be ST-23 (table 1.) We also used 5 isolates coming from bulk tank milk from dairy farms in Denmark. (table 1.) The 5 bulk milk isolates also belonged to ST-23. Two of these isolates were from farms with chronic *S. agalactiae* mastitis problems, and may belong to bovine strains. The other 3 isolates came from farms where the problem was not described as "chronic" and may belong to human strains (Jensen 1982).
Fragments from nine housekeeping genes (previously used by Sorensen 2010) were amplified (infB, soda, pgm, scpB, gdhA, dnaG, murC, polC and hexA) and PCR mix was set up as shown for species confirmation PCR (table 2.). PCR products were run as previously described and were purified using a QIAquick PCR Purification Kit(QIAGEN,Inc.,Chatworth, California, USA (Catalogue number: 28106, Lot number: 139274614) following enclosed instructions (appendix 2.). 1.5 µl of each sample was tested by Nanodrop (ND-1000 Spectrophotometer) to ensure that a sufficient quantity and quality of DNA was present after purification. Nanodrop also allowed calculation of the dilutions required for submission for sequencing. Samples of DNA and primers were then sent away for sequencing to Eurofins MWG Operon (Ebersberg, Germany). DNA sequence data obtained were proof-read and aligned by applying the Clustal W algorithm using the software programs SeqMan and MegAlign (DNA Star, Madison, Wisconsin, USA). Alleles were then assigned to each of the isolates for the 9 loci (if products were obtained by PCR) using final sequence data and the Basic Local Alignment Tool (BLAST) program on the internet.

Serotyping

For serotyping, isolates MRI Z1-208, MRI Z1-595 and MRI Z1-709 and all isolates from Denmark (table 1.) were used. Primers for serotype Ia were described by Poyart and colleagues (2007) and primers for serotype III by Kong and colleagues (2005) (table. 2.). PCR products were run on a 1.5% agarose gel as previously described and where then purified, 'nanodropped', sent for sequencing and proof-read as described for the 9 housekeeping genes. We used nucleotide BLAST to ensure products were fragments of capsular types and either capsular type Ia or III was then assigned based on PCR and sequencing results.

Virulence gene screening Gradient PCR

In order to specify PCR conditions for all virulence genes primers (table 2.) we ran a gradient PCR with isolate - MRI Z1-207 (ST67) and 2 primer sets (ABC and XRE). Annealing temperature range and primers were chosen based on information about the annealing temperature of the different primers by Eurofins. ABC was thought to have the highest annealing temperature and XRE was thought to have the lowest annealing temperature. Isolate MRI Z1-207 was chosen because it belongs to the same ST as the isolate that was used as the basis for primer design (Richards et al., 2011). Positive controls for the virulence gene PCR protocols were not available, and an isolate belonging to ST67 was considered to be most likely to yield a PCR product if the PCR conditions were correct. PCR products were ran on a 1.5% agarose gel as shown for confirmation PCR. Identity of the PCR amplicons was confirmed by DNA sequencing as described below.

Virulence gene screening PCR

Isolates used for virulence genes screening belonged to ST-23, ST-1 and ST-103 (table 1.). ST-23 is commonly found in wide range of animals including; humans, cattle, rodents, crocodiles and sea mammals, but are thought to be from human origin (Bisharat 2004, Bishop 2007, Pollock 2010). ST-1 is thought to be from human origin (Bisharat 2004). Origin of ST-103 is still unknown. Amongst isolates found on Danish dairy farms, these three sequence types were most frequently found (Zadoks et al., 2011). We used 5 isolates for each sequence type (table 1.). Originally we started testing 6 isolates (2 isolates for each sequence type) with all primers. For primer sets that yielded PCR products, we tested 3 additional isolates per sequence type. These primers are marked with an * (table 2.). PCR products were run on a 1.5% agarose gel as previously described, but products for DNA and NRP were run with a 25 bp. marker for 90 minutes because of the small amplicon size. A few products were then purified, nanodropped, sent for sequencing and proof-read as described for the 9

housekeeping genes. We used nucleotide BLAST to ensure products were fragments of virulence genes described in table 2.

Results

Species Confirmation PCR

All samples were confirmed as *S. agalactiae,* due to successful amplification of the 16s to 23s spacer regions (Phuektes et al., 2001).

Nine housekeeping genes MLST Gradient PCR

All three primer sets showed clear bands with isolate MRI Z1-202. SodA showed clear bands up until an annealing temperature of 63.9°C and a faint band for 66.8°C. DnaG showed clear bands up until 63.9°C. HexA showed clear bands up until 66.8°C and a faint band for 68.7°C. We chose to set the annealing temperature a little lower (58°C) than the maximum temperature that showed product bands, because we wanted to make sure conditions were sensitive enough for annealing to take place.

Nine housekeeping genes MLST

As expected for housekeeping genes, most isolates showed products bands for all nine primer sets. Only in a few cases no PCR products were obtained and sometimes an extra product band was seen (mainly using primers for gdhA and hexA) (fig. 4). In case extra product bands were seen, PCR product purification was done using a QIA quick Gel Extraction Kit. With this method, DNA bands are cut out from the agarose gel. This allowed us to cut out and purify products with the right size (appendix 4). After running a PCR and purifying, the DNA was sent for sequencing. All products were then assigned an allele letter or number using nBLAST (table 3).

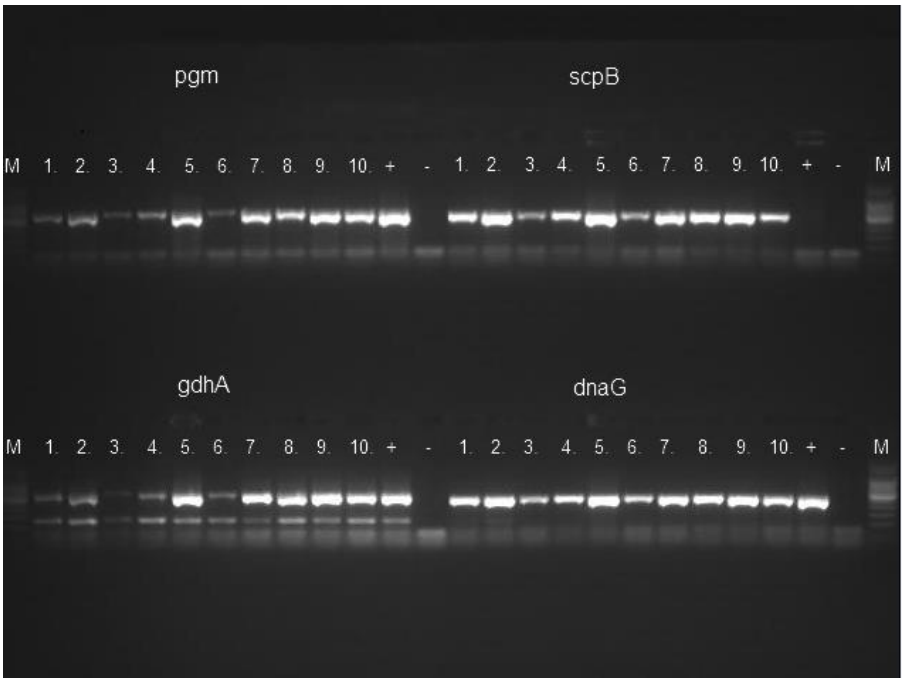

Figure 4. Housekeeping genes pgm, scpB, gdhA and dnaG PCR, M=100 bp marker, +=positive control, - =negative control, 1-10 are MRI Z1-197, -199, -200, -201, -202, -203, -204, -208, -595, -709

Table 3. Results for 9 housekeeping MLST and serotyping

Serotyping PCR

Among the 9 isolates examined, 7 isolates were assigned to serotype Ia by PCR (fig. 5, table 3). Although faint product bands (when tested with primer for cps Ia) were seen for isolates MRI Z1-060 and MRI Z1-123 these 2 isolates were assigned to serotype III by PCR, because bands in the CPSIII PCR were much stronger than those in the CPS Ia PCR (fig. 5). All PCR products were also assigned a serotype using nBLAST. Reference strains from Kong (2002) were used (fig. 7) and sequences from Ia and III (part H) were then compared to our sequences using MegAlign (fig 8). Eventually MRI Z1-060 and MRI Z1-123 were assigned serotype III-2 (table 3).

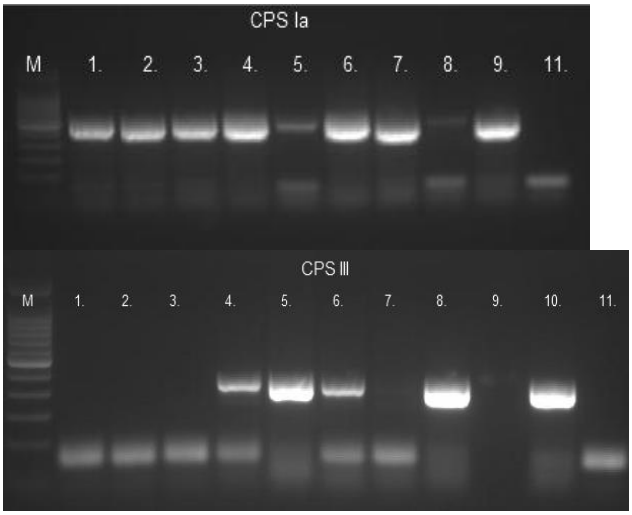

Figure 5. PCR results for Capsular serotype Ia and III, M=100 bp marker, 1-8 are MRI Z1-208, -595, -709, -024, -060, -071, -100, -123, 9 = positive control, 10 = positive control, 11 = negative control

Laboratory strain panel and strain number[a]	Source	Serotype	MS andsubtype	GenBank accession no.
Reference panel 1				
090	Channing	Ia	Ia	AF332893
H36B	Channing	Ib	Ib	AF332903
18RS21	Channing	II	II	AF332905
M781	Channing	III	III-2[b]	AF332896
3139	Channing	IV	IV	AF332908
CJB 111	Channing	V	V	AF332910
SS1214	Channing	VI	VI	AF332901
7271	Channing	VII	VII	AF332913
JM9 130013	Channing	VIII	VIII	
Reference panel 2				
NZRM 908 (NCDC SS615)	ESR	Ia	Ia	AF332894
NZRM 909 (NCDC SS618)	ESR	Ib	Ib	AF332904
NZRM 910 (NCDC SS700)	ESR	Ia	Ia	AF332914
NZRM 911 (NCDC SS619)	ESR	II	II	AF332906
NZRM 912 (NCDC SS620)	ESR	III	III-3[b]	AF332897
NZRM 2217 (Prague 25/60)	ESR	Nontypeable (R)	II	AF332907
NZRM 2832 (Prague 1/82)	ESR	IV	IV	AF332909
NZRM 2833 (Prague 10/84)	ESR	V	V	AF332911
NZRM 2834 (Prague 118754)	ESR	VI	VI	AF332902

[a]The strains in reference panel 1 were supplied by Lawrence Paoletti, Channing Laboratory. Reference panel 2 consisted of New Zealand Reference Medical Culture Collection strains, supplied by Diana Martin, ESR.
[b]The numbers indicate MS III subtypes based on sequence heterogeneity; see the text for more detail.

Figure 6. Reference strains for cps (Kong 2002)

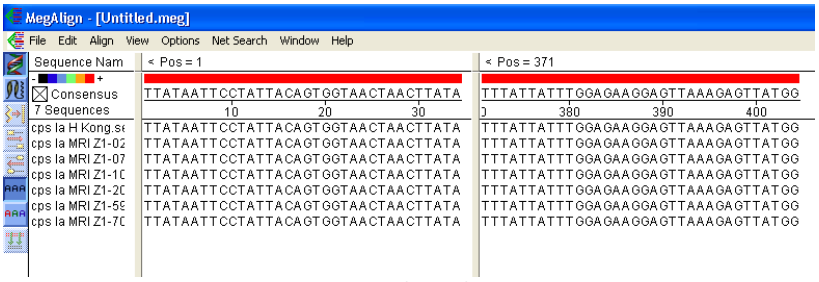

Figure 7. MegAlign: sequences from Ia and III (part H) were compared to our sequences

Virulence genes Gradient PCR

XRE did not show product bands for the expected amplicon size. It did show very faint products of a bigger size. ABC showed clear product bands up until 59.1°C (fig. 9).
We choose to set the annealing temperature a little lower (56.6°C) than the maximum temperature that showed product bands, because we wanted to make sure conditions were sensitive enough for annealing to take place.

Virulence genes PCR

Because primers CTP, XRE and PDS did neither show products bands for the isolates nor for the positive control, we tested all isolates on the 5 remaining primer sets only. Isolate MRI Z1-180 did not show a product band for any of the primers we used, but all other isolates showed 1 or more product bands (table 4).

Isolate	ST	ABC	DNA	NRP	LAC	RDP
MRI Z1-024	ST23	-	-	-	-	+
MRI Z1-060	ST23	+	+	-	-	-
MRI Z1-071	ST23	-	-	+	+	+
MRI Z1-123	ST23	-	-	+	+	-
MRI Z1-165	ST23	-	-	-	-	+
MRI Z1-025	ST1	-	-	+	+	+
MRI Z1-054	ST1	-	+	+	+	+
MRI Z1-073	ST1	-	-	-	-	+
MRI Z1-101	ST1	+	-	-	-	-
MRI Z1-180	ST1	-	-	-	-	-
MRI Z1-023	ST103	+	-	+	-	-
MRI Z1-084	ST103	+	-	-	-	-
MRI Z1-087	ST103	+	+	-	-	-
MRI Z1-169	ST103	+	-	-	-	-
MRI Z1-226	ST103	+	-	-	-	-

Table 4. Results for virulence genes screening, cells highlighted in green represent products bands for these isolates with the specific primers. Four other primers we used are not shown in this table as they did not show any products (neither for pos. control).

Discussion

MLST using 7 housekeeping genes is routinely used for molecular typing of *S. agalactiae*. Because it is a standardized method, MLST results can be compared across studies. Based on numerous studies of *S. agalactiae* of humans and animals, certain STs have been identified as primarily human-associated, e.g. ST1, ST17 and ST19 (Jones et al., 2003), whereas other STs have been identified as primarily bovine-associated, e.g. ST67 (Bisharat et al., 2004). ST23 has been associated with both host species. An expanded MLST scheme has been developed to provide further differentiation of ST23 isolates. This expanded MLST scheme, using an additional 9 housekeeping genes, allows for identification of human-associated and bovine-associated subpopulations of ST23 (Sorensen et al., 2010). Comparing our results with the results Sorensen found leads to the conclusion that most isolates we used for our study belonged to Group G described by Sorensen (Sorensen et al. 2010). Sorensen found that Group G comprises mostly human isolates (n=17) and to a lesser extend bovine isolates (n=3). In our study both sea-mammal/dog isolates from Scotland and bovine isolates from Denmark were included. This was thought to increase the chances to see variety between alleles obtained with expanded MLST scheme and to find both isolates that originated from human – and bovine subpopulations of ST23. Because of previous studies (Yildirim et al. 2002, Pollock 2010) we expect that the canine- and sea mammal isolates originate from human strains. We expected the isolates that were obtained from bulk tank milk from Danish dairy farms to come from bovine strains. Results obtained by MLST show that all sea mammal isolates most likely came from human strains and most dairy isolates from Denmark are also likely to come from a human strain, although two of these isolates showed slight differences in MLST compared to the other isolates. Results from this part of the study suggest that re-emergence of *S. agalactiae* may be due to a host-species jump of human strains, rather than to re-emergence of strains that were historically associated with cattle.

In addition to MLST, capsular serotyping can be used for strain typing of *S. agalactiae*. Specific serotypes are associated with specific host species, particularly within ST23. Capsular type Ia is primarily associated with human ST 23 and capsular type III bovine is primarily found in bovine strains of ST23 (Sorensen et al. 2010). Serotyping results showed that all sea-mammal/dog isolates had capsular type Ia. Most bovine isolates also had capsular type Ia, but two of them had capsular type III. These isolates were also slightly different compared to the other isolates on the expanded MLST scheme. Although MLST showed that they likely belonged to group G, which is thought to originally come from human strains, the serotyping results show that they had capsular type III which belongs to bovine strains. We think that the expanded MLST scheme results using 9 alternative housekeeping genes are not showing enough differentiation between isolates and based on slight differences in the expanded MLST and the serotyping results, we think that these isolates are of bovine origin. It is described that isolates with capsular type III are difficult to type using serotyping (Bisharat et al. 2004), this also applied for our type III isolates. With nBLAST we compared DNA-sequences for amplicons from the serotyping PCR results with reference strains from Kong et al. (2002). We specifically focused on the cpsH gene, because this is the element in the type Ia and III capsular operon that differentiates between them. We found that our type III isolates were all type III-2.

Richards compared the whole genome from 1 bovine *S. agalactiae* isolate with 8 whole genomes from human *S. agalactiae* isolates (Richards et al. 2011). He found that particular virulence genes are much more commonly (or exclusively) found in bovine isolates (fig. 3). We hypothesized that acquisition of virulence genes by human derived strains of *S.agalactiae* might give them a survival advantage in cattle. An example of such genes is the lac operon, which encodes for lactose fermentation and may be essential for survival in the bovine udder. This hypothesis was explored for a selection of ST23 isolates, as well as for ST1 isolates and ST103 isolates because those are the types that are most commonly found in Denmark (Zadoks et al., 2011). Like ST23 serotype 1a, ST1 is primarily associated with humans. The origin of ST103 is unknown. We expected that all isolates at least had this lac operon as they came from bulk tank milk from dairy cows and possibly would also have other virulence genes. We found that not all isolates had the lac operon. This could be due to another lac operon somewhere else in the genome. It is known that S. canis has another lac operon (Richards et al., submitted for publication), it could be possible that our *S. agalactiae* isolates had this lac operon. The nrp operon and lac operon were present in the same isolates, which could be due to their place in the genome on the same genomic island (Richards et al., 2011). Although this does not seems to be the case with all operons, as the rdp and abc operon are on the same genomic island but not present in the same isolates. It was seen that all ST103 isolates had the abc operon but not nrp operon and most ST1 and ST23 had the nrp operon but not abc operon. None of the genes seemed to be essential for the survival in the bovine udder, but it is likely that

having one or more of these genes would give the isolates a survival advantage. The functional mechanism behind these virulence genes is unknown. Our data showed that isolates that originated from a human strain adapted to the cow as a different host species, possibly through Lateral Gene Transfer of virulence genes.

Conclusion and Outlook

Non-specific primer binding was observed for MLST, Serotyping and Virulence gene screening PCR, this showed us that the protocols that we used do not always perform the way you expect.
We found that MLST and serotyping results suggest that all sea-mammal/dog isolates belonging to ST23 came from human strains. Most bovine isolates also came from humane strains, although MLST and serotyping results were not consistent in the case of two bovine isolates.
None of the bovine isolates of ST1, ST23 or ST103 acquired all virulence genes and most isolates did not have the lac operon. This operon is thought to be essential for survival in the bovine udder. In a subsequent project , those *S. agalactiae* were shown to grow in lactose broth, proving that they have the ability to ferment lactose even when they did not have the lac operon for which we tested by PCR. One of the possible explanations for the absence of this operon could be that these isolates have a different mechanism to ferment lactose, which could possibly be the same lac operon as *S. canis.*
Whole genome sequencing would provide more insight in host adaptation of human and bovine *S. agalactiae* from bulk tank milk from Danish dairy farms. Also looking at individual cows and farm management practice could provide more insight in spread and clinical signs of different *S. agalactiae* isolates.

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Appendices

Appendix 1. Method of making bacterial cultures

1. Ten µl was taken for each isolate from a frozen glycerol stock and cultured onto a sheep blood agar plate and incubated at 37°C for 24 hours.
2. One colony was then taken from the plate and immersed in Brain Heart Infusion (BHI) broth and incubated at 37°C overnight.

Appendix 2. Boiling method for extraction of DNA

1. 1.2 ml of overnight growth suspension in BHI was centrifuged at 14,000 rpm for 8 minutes.
2. The supernatant was removed and 1 ml of TE Buffer was added to the remaining pellet and centrifuged at 14,000 rpm for 1 minute.
3. The supernatant was removed and the pellet was resuspended in 100 µl of TE Buffer.
4. Mixture was then incubated in a heat block at 95°C for 10 minutes to lyse the cells
5. The lysates were cooled on ice and centrifuged at 14,000 rpm for 1 minute to remove cellular debris.
6. The pellet was removed and the supernatant was transferred to a new Eppendorff tube and stored at -21°C.

Appendix 3. QIAquick PCR Purification Kit Protocol

1. Add 5 volumes of Buffer PB to 1 volume of PCR sample and mix
   Colour of the mixture should be yellow, confirming the correct pH. If colour is orange or violet, add 10 µl of 3 M sodium acetate (pH 5) and mix.
2. Place a QIAquick spin column in a 2ml collection tube.
3. To bind DNA, apply the sample to the column and centrifuge for 60 seconds at 10,000 rpm in a Sigma 1-15 microcentrifuge (Sigma Aldrich, USA).
   Discard flow through and place the column back in the collection tube.
4. To wash, add 0.75 ml of Buffer PE to the column and centrifuge for 60 seconds at 10,000 rpm.
5. Discard the flow through and centrifuge the column for 1 minute at 10,000 rpm.
6. Place each QIAquick column in a clean 1.5 ml microcentrifuge tube.
7. To increase DNA concentration add 30 µl water to the center of the QIAquick membrane and centrifuge the column for 1 minute.

Appendix 4. QIAquick Gel Extraction Kit Protocol

1. Excise the DNA fragments from the agarose gel with a clean and sharp scalpel. Minimize the size of the gel slice by removing extra agarose.
2. Weigh the gel slice in a colorless tube. Add 3 volumes of buffer QG to 1 volume of gel (100 mg-100µl). For gel slices more than 400 mg use more than 1 QIAquick column.
3. Incubate the samples at 50°C for 10 minutes (or until gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2-3 minutes during the incubation.
4. After the gel slice has dissolved completely add 1 gel volume of isopropanol to the sample and mix. This step increases the yield of DNA fragments <500 base pairs.
5. Place a QIAquick spin column in a provided 2 ml collection tube.
6. To bind DNA apply the sample to a QIA quick column and centrifuge for 1 minute.
7. Discard the flow through and place the QIA quick column back in the same collection tube.
8. Add 0.5 ml of Buffer QG to a QIA quick column and centrifuge for 1 minute, This step will remove all traces of agarose.
9. To wash add 0.75 ml of Buffer PE to QIA quick column and centrifuge for 1 minute. Let column stand for 2-5 minutes before centrifuging.
10. Discard the flow through and spin for an extra minute.
11. Place column into a clean 1.5 ml microcentrifuge tube and add 30 µl of water to the center of the membrane. Let the column stand for 1 minute and then centrifuge for 1 minute.

Appendix 5. Growing SAG in milk

All strains were bovine isolates from bulk tank milk form Danish dairy farms.

MRI Z1-025	ST1
MRI Z1-073	ST1
MRI Z1-060	ST23
MRI Z1-123	ST23
MRI Z1-023	ST103
MRI Z1-084	ST103
MRI Z1-207	ST67

1. All isolates were inoculated from a frozen stock onto a sheep blood agar plate (BAP) and left overnight at 37 °C. Then agar plates were put in a cold room at 4°C for three days. Using sterile plastic loops, 1 colony of each isolate was then put from the BAP into a bijou with BHI 15ml. And left in a hot room at 37°C for four hours. Also a negative control; using a sterile loop inoculating BHI without a colony on the loop, was added and left at 37°C for four hours.
2. After four hours a dilution series was made from the BHI. Putting a 100µl of BHI with isolate in the first well on a 96 well plate. And each next well was made into a 1:10 dilution of the previous one, to a 10^{-5} dilution.
3. After making dilution series the dilutions were put on a BAP. Dividing each BAP in three regions, using two plates per isolate.
4. The BAP were then dried in a hotroom at 37°C for two hours prior to placing drops of the dilutions on them. This was done because otherwise dilutions would run off the plate. All plates were left overnight at 37°C. Dropsize we used was 20 µl
5. Ten µl of BHI was also inoculated in milk. We used skimmed 0,1% fat commercially sold pasteurized milk. We used both boiled and unboiled milk to see if there was any difference between growth of the isolates and also to see if any other bacteria were present in the unboiled milk versus the boiled milk

(we used two bijous with milk, per bijou BHI and also added another negative control for the milk, in total there were 16 bijou's with 10 µl).

6. Also one colony of each isolate was inoculated directly into the milk, for this we only used boiled milk.
7. We left all bijou's with milk overnight at 37°C.

Dilution series in BHI Results

All isolates showed strong growth, even at the highest dilution most isolate colonies could not be counted because there were too many. Remarkably isolate MRI Z1-207 did not grow as well as other isolates and colonies could be counted at a 10^{-5} dilution while MRI Z1-207 was included as a positive control because it belongs to ST67, a bovine associated sequence type know to have the *lac* operon. Because of these results, we decided to make higher dilutions up to 10^{-7}. Although we were expecting that bacteria will not grow as well in milkas they do in BHI, we still decided to make higher dilutions because bacteria were left in the milk for fourteen hours, which was much longer than how long they were left in BHI (four hours).
We divided plates up in four regions instead of three and used smaller drops of 10µl instead of 20µl. We left all plates overnight at 37°C.

All plates showed contamination. Apart from *S.agalactiae* we found 'mucus like' colonies, white 'fungi like' material and bacteria that formed colonies with either α-hemolysis or β-hemolysis. It was difficult to count single colonies, but with higher dilutions we foundless other bacteria and very likely only saw *S. agalactiae*.

Isolate	**Concentration in boiled milk**	**Concentration in unboiled milk**	**BAP bacteria in boiled milk**
MRI Z1-025	433×10^6	400×10^5	1600×10^6
MRI Z1-084	866×10^6	433×10^6	1600×10^6
MRI Z1-060	1166×10^6	400×10^7	500×10^7
MRI Z1-123	666×10^6	400×10^5	2000×10^7
MRI Z1-023	733×10^6	2850×10^7	500×10^6
MRI Z1-073	666×10^6	1333×10^7	633×10^6

All negative controls showed contamination.Earlier results (Tassi unpublished) show that *S. uberis* grows better in boiled milk than in unboiled milk. Here we see that *S. agalactiae* overall grew better in unboiled milk. Also we saw that bacteria that were brought straight from the BAP into the milk instead of being put in BHI first grew slightly better. We did not expect this, as bacteria need time to accumulate and get used to a certain environment. We expected that bacteria that were brought in BHI first could improve this characteristic and would therefore grow better in the milk.
Something that we have to take into account is that we left the bacteria for fourteen hours in the milk and by this time all bacteria would have probably reached their stationary phase in growth.
We did not expect to see this much contamination, especially not in the boiled milk. There are several moments in time were contamination could have taken place:

1. It was already present in the milk and pasteurization did not kill all bacteria
2. Before boiling (considering temperatures were not high enough or a high temperature was not maintained);

- When milk was put in a sterile bottle
- Sterilizing the bottle was not done in a proper way
- When boiling the milk, magnet that was put in was not sterile

3. When applying bacteria from BHI or BAP into the milk
4. When applying drops of milk to BAP
5. BAP were not sterile

References

- Bisharat, 2004 N. Bisharat, Hyperinvasive neonatal Group-B *Streptococcus* has arisen from a bovine ancestor, *J. Clinical Microbiology* 42 (2004), pp. 2161-2167
- Bishop et al., 2007 EJ. Bishop, Necrotizing fasciitis in captive juvenile *Crocodylus porosus* caused by *Streptococcus agalactiae*: an outbreak and review of the animal and human literature, *Epidemiology and Infection* 135 (2007), pp. 1248-1255
- Bradley et al.,2007 AJ. Bradley, Survey of the incidence and aetiology of mastitis on dairy farms in England and Wales, *Veterinary Record* 160 (2007), pp. 253-258
- Brochet, 2006 M. Brochet, Genomic diversity and evolution within the species *Streptococcus Agalactiae*, *Microbes and Infection* 8 (2006), pp. 1227-1243
- Chaffin, 2000 D. Chaffin, The serotype of type Ia and III Group B *Streptococci* is determined by the polymerase gene within the polycistronic capsule operon, *J. Bacteriology* 182(16) (2000), pp. 4466-4477
- Cieslewicz, 2005 CJ. Cieslewicz, Structural and genetic diversity of group B streptococcus capsular polysaccharides., Infect Immun. 5 (2005), pp. 3096-30103
- Delannoy –unpublished-Moredun
- Dogan, 2005 B. Dogan, Distribution of serotypes and antimicrobial resistance genes among *Streptococcus agalactiae* isolates from bovine and human hosts, *J.Clinical Microbiology,* Dec. 2005, pp. 5899-5906
- Enright, 1999 MC. Enright, Multilocus sequence typing, *Trends in microbiology* vol. 7 no. 12 (1999), pp.
- Evans, 2008 JJ. Evans, Phylogenetic relationships among *Streptococcus agalactiae* isolated from piscine, dolphin, bovine and human sources: a dolphin and piscine lineage associated with a fish epidemic in Kuwait is also associated with human neonatal infections in Japan, *J. Medical Microbiology* 57 (2008), pp. 1369-1376
- Feil, 2004 EJ. Feil, eBURST: Inferring Patterns of Evolutionary Descent among Clusters of Related Bacterial Genotypes from Multilocus Sequence Typing Data, *J. Bacteriology*, March 2004, pp. 1518-1530
- Jensen, 1980 NE. Jensen, Distribution of serotypes of Group-B *Streptococci* in herds and cows within an area of Denmark, *Acta veterinaria Scandinavica* 21 (1980), pp. 354-366
- Jensen, 1982 NE. Jensen, Experimental bovine Group-B streptococcal mastitis induced by strains of human and bovine origin,*Nordisk Veterinaermedicin* 34 (1982), pp. 441-450
- Jones, 2003 N. Jones, Multilocus Sequence Typing System for Group B Streptococcus, *J. Clinical Microbiology* June (2003), pp. 2530-2536
- Katholm, 2010 J. Katholm, *Streptococcus agalactiae* – an increasing problem in Scandinavia, NMSM, Mastitis symposium 9th June 2010
- Keefe, 1997 GP. Keefe, *Streptococcus agalactiae* mastitis: a review, *Can. Vet. J.* 38 (1997), pp. 429-437
- Kong, 2005 F. Kong, Simultaneous detection and serotype identification of *Streptococcus agalactiae* using multiplex PCR and reverse line blot hybridization, Journal of Medical Microbiology 54 (2005), pp. 1133-1138
- Lachenauer , 1999 CS. Lachenauer, Serotypes VI and VIII predominate among group B streptococci isolated from pregnant Japanese women., J Infect Dis. 4 (1999), pp. 1030-1033
- Loeffler, 1995 SH. Loeffler, The dairying veterinary approach to a high bulk milk cell count caused by *Streptococcus agalactiae*, *Tijdschrift voor diergeneeskunde* 120 (1995), pp. 458-463
- Mhalu, 1976 FS. Mhalu, Infection with *Streptococcus agalactiae* in a London hospital, *J. Clinical Pathology* 29(1979), pp. 309-312
- Mian, 2009 GF. Mian, Aspects of the natural history and virulence of *S. agalactiae* infection in Nile tilapia., *Vet Microbiology* 14 (2009), pp. 136-180
- Pollock, *Streptococcus agalactiae* in Sea Mammals: Human, Bovine or Piscine?–unpublished- Moredun
- Poyart, 2007 C. Poyart, Multiplex PCR assay for rapid and accurate capsular typing of group B streptococci., J Clin Microbiol. 6 (2007), PP. 1985-1988
- Richards, 2011 VP. Richards, Comparative genomics and the role of lateral gene transfer in the evolution of bovine adapted *Streptococcus agalactiae, Infection Genetics and evolution* 11 (2011), pp 789-801
- Sorensen et al., 2010 UB. Sorensen, Emergence and global dissemination of host specific *Streptococcus agalactiae* clones, *Mbio* 24 (2010), pp. e00178-10
- Spratt, 1999 BG. Spratt, Multilocus sequence typing: molecular typing of bacterial pathogens in an era of rapid DNA sequencing and the internet, *Current opinion in microbiology* 2 (1999), pp. 312-316
- Sukhnanand, 2005 S. Sukhnanand, Molecular subtyping and characterization of bovine and human *Streptococcus agalactiae* Isolates, *J. Clinical Microbiology,* March 2005, pp. 1177-1186
- Yildirim, 2002 AO. Yildirim, Pheno- and genotypic properties of *streptococci* of serological group B of canine and feline origin., FEMS Microbiol Lett. Jul 2 (2002), pp. 187-192.
- Zadoks, Danish presentation-unpublished-