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Molecular Methods for Typing of *Streptococcus agalactiae* with Special Emphasis on the Development and Validation of a Multi-Locus Variable Number of Tandem Repeats Assay (MLVA)

Thesis for the degree of Philosophiae Doctor

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Norwegian University of Science and Technology
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Molekylære metoder for typing av *Streptococcus agalactiae* med særlig vektlegging av utvikling og validering av et multi-locus variable number of tandem repeats assay (MLVA)

Sammendraget:
Streptococcus agalactiae eller gruppe B streptokokker (GBS) forårsaker livsfarlige infeksjoner hos nyfødte, gravide eller voksne med kroniske sykdommer. Den forårsaker også jurbetennelse i storfe. Typing av GBS gir innblikk i bakteriens epidemiologi og dens fylogenetiske slektskap. Ulike deler av bakteriene kan være mål for typingsmetoder. Eldre immunologiske metoder fokuserer ofte på overflateegenskaper som polysakkarid- eller proteinstrukturer. Nyere molekylære metoder benytter bakteriens genmateriale til typing.
Studien undersøkte om molekylære metoder hadde potensiale til å gi en bedre oppløsning av en stammesamling. I detalj ble typingen av overflateproteiner med både immunologiske og molekylære metoder sammenlignet og en multi-locus variable number of tandem repeats assay (MLVA) ble utviklet og evaluert. Sistnevnte metode er basert på variabiliteten i repeterte områder i bakteriens genom.
Sammenligning av sero- og genotyping av GBS overflateproteiner er kompleks på grunn av kryssreaksjoner mellom de ulike proteinene som er sammensatt fra "samme byggesett". Positive resultat for begge metoder ble funnet for 122 av 147 stammer. Av disse hadde 74 % overensstemmende resultater. Ikke overensstemmende resultater ble funnet for tre og delvis overensstemmede resultater for 29 stammer.
Utvikling av en MLVA for GBS ble gjort gjennom analyse av publiserte, helgenomer for tre stammer som resulterte i testing av i alt 18 kandidatloci. Videre undersøkelser identifiserte fem loci som ble inkludert i studiens MLVA.
En stammesamling av 126 stammer fra nyfødte ble delt inn i 70 grupper av MLVA metoden, noe som representerte en klart overlegen oppløsning sammenlignet med to referansemetoder. Videre ble metodens egnethet for typing av epidemiologisk relaterte stammer demonstrert ved å undersøke 187 stammer som hadde forårsaket jurbetennelse hos storfe. Stammene var samlet inn fra 34 gårder og det ble funnet 37 typer, stort sett en type per gård. På en gård som var representert med 48 stammer ble en forandring av et av MLVA områdene under innsamlingsperioden observert og kan gjenspeile stabiliteten av repeterte områder under in-vivo forhold.
Oppsummert ble det vist at immunologiske og molekylære metoder viser overensstemmende eller delvis overensstemmende resultater i det store flertall av stammer. Molekylære metoder er overlegen i typingssammenheng siden det fører til mindre tvetydighet. MLVA metoden for GBS fungerte eksellent i studien og viste veldig god evne til å skille stammene i epidemiologisk relaterte grupper.

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Collaboration was essential for all three papers. The first paper is the result of collaboration with the Gilbert group in Sydney. Fanrong Kong was my invaluable partner Down Under for the practicalities of the realization of the laboratory work, analysis of results and article writing. Gwendolyn Gilbert is acknowledged especially for her help during the writing of the manuscript, in particular when we were forced to reduce the length of the manuscript by one third. Throughout the whole process from planning to the writing of Paper II Bjørn-Arne Lindstedt's extensive knowledge of and experience with the MLVA method were invaluable. For Paper III Torkjel Bruheim was responsible for collecting bovine GBS strains over several years and having them ready for the serendipitous match of this collection with our newly designed MLVA. It has been an immense pleasure both to work with and getting acquainted to him.

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List of papers

I. Radtke A, Kong F, Bergh K, Lyng RV, Ko D, Gilbert GL. Identification of surface proteins of group B streptococci: serotyping versus genotyping. Journal of Microbiological Methods. 2009 Sep;78(3):363-5.

II. Radtke A, Lindstedt BA, Afset JE, Bergh K. Rapid multiple-locus variant-repeat assay (MLVA) for genotyping of *Streptococcus agalactiae*. Journal of Clinical Microbiology. 2010 Jul;48(7):2502-8.

III. Radtke A, Bruheim, T, Afset JE, Bergh K. Multiple-locus variant-repeat assay (MLVA) is a useful tool for molecular epidemiologic analysis of *Streptococcus agalactiae* strains causing bovine mastitis. Veterinary Microbiology. 2012. Available online: http://dx.doi.org/10.1016/j.vetmic.2011.12.034. In press.

Summary

Streptococcus agalactiae or group B streptococcus (GBS) is a commensal organism in humans but can cause life threatening infection in susceptible hosts such as neonates, pregnant women and non-pregnant adults with chronic illnesses. It is also a cause of mastitis in bovines. Typing of GBS is performed to gain insight into the epidemiology and the phylogeny of the organism. Numerous typing methods have been used over the past 80 years reflecting the technical possibilities of their time. Over the past 20 years molecular methods have become common.

Typing of GBS usually starts with the determination of the capsular polysaccharides (CPS). Subtyping of strain variable surface proteins is performed by some investigators. These proteins consist of the alpha-like proteins Cα, Alp1, Alp2, Alp3, Alp4 and R4 and the Cβ protein. Also other proteins such as the R3 protein can be used for subtyping. This typing has traditionally been performed by immunological methods.

Molecular typing methods have several advantages over serotyping, among them the generation of more unambiguous results and they bypass the problem of immunological cross-reactivity. More advanced molecular methods have the ability to differentiate strain collections into many types. Examples of this are pulsed-field gel electrophoresis and multi-locus sequence typing (MLST).

This study aimed at investigating the potential of molecular methods for better resolution for the typing of GBS. Specifically the typing of surface proteins by immunological and molecular methods was compared and a multi-locus variable number of tandem repeats assay (MLVA) was developed and investigated.

Methods used in the study were serotyping of surface proteins, genotyping of surface proteins and capsular polysaccharides, MLST and MLVA. The GBS strain collections used consisted of 147 and 126 human strains in paper I and II, respectively, and 187 bovine strains in paper III.

The comparison of sero- and genotyping of GBS surface proteins is complex due to the mosaicism of the alpha-like proteins which results in cross-reactivity. Of the 147 isolates used in paper I 24 and one were non-typable by sero- and genotyping, respectively. The two methods produced congruent results in 73.8% of 122 strains which were typable by both methods, discordant results in three and partially discordant results in 29 strains.

The construction of a MLVA was possible through in-silico screening of the genomes of three fully sequenced strains followed by the construction and analysis of PCRs for 18

candidate loci. Five of these loci were selected for the proposed MLVA. The MLVA generated clusters which corresponded well with those observed by the two other methods but provided a considerably higher degree of diversity. The strain collection of 126 strains was divided into 70 types by MLVA, 36 by MLST and 19 by the combination of CPS and surface proteins. The strains were clustered into comparable groups.

To demonstrate the suitability of the MLVA method for high resolution typing of epidemiologically related strains we investigated 187 bovine strains. The strains were collected at 34 farms. MLVA analysis divided this strain collection into 37 types. In 29 farms all GBS strains had identical MLVA profiles specific for each farm. In one farm represented with 48 isolates, four MLVA variants with differences in one repeat locus were observed during the collection period of almost three years. Similar variations were observed at four other farms. This might reflect the stability of repeat loci under in vivo conditions.

In summary the study showed that typing of GBS surface proteins by immunological and molecular methods provides concordant or partially concordant results for the large majority of strains. Genotyping is superior to serotyping in this setting since it is able to type almost all strains and leads to less ambiguity. The MLVA typing scheme for GBS designed as part of the study performed excellently with very good discrimination. MLVA typed the strains into epidemiological groups comparable to MLST and typing of CPS and surface proteins. MLVA analysis of bovine GBS allocated a specific genotype to almost every farm while isolates from one farm were always identical or closely related. Taken together the results indicate that the MLVA is highly applicable for elucidating epidemiological relationships in GBS.

Abbreviations

AFLP	Amplified restriction fragment length polymorphism
Alp	Alpha like protein
AMS	Automated milking system
ATCC	American Type Culture Collection
bp	Base pair
CAMP-test	Christie, Atkins, Munch-Petersen test
CC	Clonal complex, group of highly related MLST types
CI	Confidence interval
CPS	Capsular polysaccharide
CRISPR	Clustered regularly interspaced short palindromic repeats
FAT	Fluorescence antibody test
GBS	Group B streptococcus
IAP	Intrapartum antibiotics prophylaxis
MLST	Multi-locus sequence typing
MLVA	Multi-locus variable number of tandem repeats assay
NVI	National Veterinary Institute
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
SATR	*Streptococcus agalactiae* tandem repeat
SLV	Single locus variants
SNP	Single-nucleotide polymorphisms
SSR	Short sequence repeat
ST	Sequence type (= MLST)
TR	Tandem repeat, used as designation in the first part of Study II
VNTR	Variable number of tandem repeats

1 Introduction

Streptococcus agalactiae or group B streptococcus (GBS) is a commensal organism in humans but can cause life threatening infection in susceptible hosts such as neonates, pregnant women and non-pregnant adults with chronic illnesses (Schuchat et al., 2006). It is also a cause of mastitis in bovines (Keefe, 1997). Typing of GBS has provided many insights into the epidemiology of the species. Serotyping of the capsular polysaccharides (CPS) has uncovered high prevalence of serotype III in neonatal disease and the emergence of serotype V in adult disease (Blumberg et al., 1996). The surface proteins of GBS have also been studied extensively and used by some groups for adding further resolution to CPS typing. Both GBS surface structures are important targets for the ongoing development of a capsule based vaccine. Multi-locus sequence typing (MLST) which was introduced in 2003 revealed predominance of sequence type 17 among newborns infected by serotype III strains and that most GBS belong to four major clonal complexes (Jones et al., 2003). Although MLST and pulsed-field gel electrophoresis (PFGE) are valuable typing methods they have some drawbacks, being time-consuming, expensive and need expert personnel. Further MLST is not a high resolution method and PFGE has limitations in the exchange and storage of results. Multi-locus variable number of tandem repeat assays (MLVA) have in recent years been proposed for several bacterial species as methods which are faster, cheaper and easier to perform. They can have high resolution depending on the choice of loci and the numerical results are easy to exchange and store.

1.1 Group B streptococci

1.1.1 Bacteriology of GBS

Streptococcus agalactiae is a species in the genus of *Streptococcus*. *Streptococcus* is part of the family of Streptococcaceae in the order of Lactobacillales. The members of the genus are facultative anaerobic, catalase-negative gram-positive cocci. Their metabolism is mainly fermentative and lactic acid is the predominant end product (Whiley and Hardie, 2009). Streptococci divide in one plane and therefore occur as pairs or chains. Streptococci may display beta-hemolytic, alpha-hemolytic and non-hemolytic reactions on blood agar. Those with beta-hemolytic ability, i.e. to lysate erythrocytes completely in blood agar, were subdivided by their reaction to specific antisera against their group-specific cell wall anchored carbohydrate (Kilian, 2010). These tests were initially done with immunoprecipitation. The

classification of these streptococci was described by Rebecca Lancefield. In this classification *S. agalactiae* is the only species belonging to the serogroup B (Lancefield, 1934). This has led to a synonymous use of the term "group B streptococcus", abbreviated GBS for the species.

Chains are formed by GBS usually with more than 4 cells. Chains can be long, especially in liquid media and clinical material. Colonies of overnight cultures of GBS on blood agar media tend to be >0.5 mm in diameter, typical for the large-colony-forming streptococci of the pyogenic group and differentiating GBS from the minute-colony-forming streptococci. The colonies are usually grayish-white but some strains can be pigmented from yellowish to brick-red which is unique in the genus (Whiley and Hardie, 2009). A small zone of complete hemolysis around the colonies is typical. The hemolysis is in some strains only apparent when colonies are removed from the agar (Spellerberg and Brandt, 2011). The hemolysin in GBS does not appear to be a major virulence factor and is not related to the streptolysins of *Streptococcus pyogenes* (Weiser and Rubens, 1987). About 1% of GBS strains are α- or non-hemolytic (Edwards and Nizet, 2011).

The cell wall of gram-positive bacteria has interlinked layers of peptidoglycan which add up to a thickness of 15 to 30 nm. This structure gives the cell rigidity and is important in controlling the intracellular turgor. Interspersed into this skeleton are secondary polymers such as teichoic acid structures. Polysaccharides and surface proteins are bound into the peptidoglycan and several of them protrude into the extracellular space often conferring virulence as discussed below (Beveridge and Matias, 2006). Surface proteins are anchored to the cell surface in different ways, the most common way is C-terminal anchoring including a well conserved LPXTG motif which is positioned right at the outer surface of the cytoplasmic membrane (Figure 1) (Fischetti, 2006).

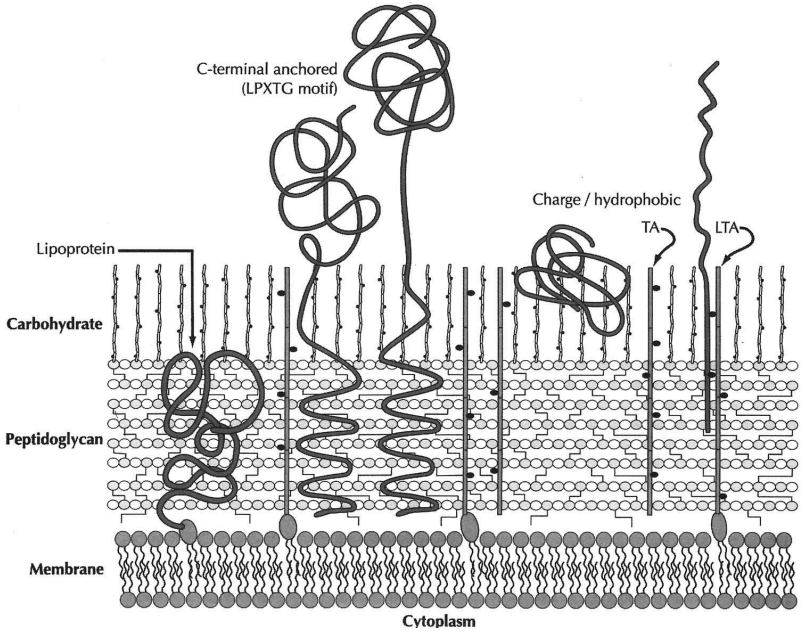


Figure 1: Major surface structures of the cell wall of gram-positive bacteria. Linked to the surface of the peptidoglycan, many gram-positive organisms have polysaccharide structures that in some cases are used for their immunological classification. Surface proteins are linked by three mechanisms. (i) Lipoproteins have a lipid linked through a cysteine at the N terminus. (ii) C-terminal-anchored proteins are attached and stabilized in the peptidoglycan through a C-terminal complex containing an LPXTG motif. (Most surface proteins are anchored in this way.) (iii) Certain surface proteins are attached through hydrophobic and/or charge interactions to the cell surface. (Some proteins are bound ionically to the lipoteichoic acid.) The teichoic acids (TA) are a common feature of the gram-positive cell wall. TA is usually composed of a repeating carbohydrate-phosphate polymer linked through a phosphodiester linkage to the peptidoglycan. Lipoteichoic acid (LTA) is composed of a similar polymer linked to the cytoplasmic membrane through a fatty acid. Figure reprinted from (Fischetti, 2006) with permission from the publishers.

Presumptive identification of streptococci from bovine mastitis was first made possible in the 1920s by analysis of sodium hippurate hydrolysis (Ayers and Rupp, 1922) and later by the above mentioned Lancefield typing system (Lancefield, 1933). The CAMP-test, introduced in 1944 (Christie et al., 1944) became later a standard test for identification of GBS. It uses the synergistic hemolytic effects of *Staphylococcus aureus* sphingomyelinase C together with a GBS co-hemolysin. Today rapid tests, which usually include antisera from the six most important Lancefield groups, are available from several suppliers, usually based on latex agglutination.

1.2.2 Short history of GBS

Historically Nocard and Mollereau were in 1887 the first to report the isolation of streptococci from bovine mastitis (Nocard and Mollereau, 1887). Several reports in the

following years described streptococci as pathogens of mastitis, among them Lehmann and Neumann in 1896 using the term *Streptococcus agalactiae* (Lehmann and Neumann, 1896). In humans Hare and Colebrook noticed the difference between hemolytic streptococci isolated from vaginal samples in parturient women with or without puerperal fever. The streptococci of from parturient women without puerperal fever resembled those found in mastitis in cattle (Hare and Colebrook, 1934). A closer investigation of these streptococci was made as mentioned above by Lancefield in her extensive research on hemolytic streptococci (Lancefield, 1933, 1934; Lancefield and Hare, 1935). This research provided the typing system for hemolytic streptococci bearing her name which is based on the group specific polysaccharides. Further research on GBS discovered the capsular polysaccharides (CPS). Initially four types of CPS were demonstrated and found to be strain specific (Lancefield, 1934, 1938). This provided the first typing system for GBS.

The initial isolations of GBS were made in cases of bovine mastitis and at that time this was seen as the main manifestation of GBS disease, despite a few reports linking GBS to puerperal septicemia (Fry, 1938; Lancefield and Hare, 1935) or invasive disease in children (Plummer, 1941; Wheeler and Foley, 1943).

The association of GBS to human disease was not properly recognized until the end of the 1950s (Eickhoff et al., 1964) and became generally accepted through the 1960s (Finn and Holden, 1970). This sudden emergence of GBS as human pathogen seems to be genuine although other developments may have had an impact. The introduction of antibiotics through the 1950s led to the disappearance of puerperal fever as the major problem of the perinatal period. This may have led to increasing awareness for other diseases. Improvements in laboratory technology may also have contributed (Ross, 1984). Before the introduction of pasteurization cow milk contaminated with bovine GBS may have contributed to the epidemiology of GBS in humans. Contemporary reviews of this development were written e.g. by Finn and Holden (Finn and Holden, 1970), Jelínkoá (Jelínkoá, 1977) and Ross (Ross, 1984).

1.2 Major antigens of GBS

1.2.1 Group B antigen

GBS has two types of surface polysaccharides, the group B antigen and type specific capsular polysaccharides of which ten have been described so far. The group antigen encountered in all GBS consists of rhamnose, N-acetyl glucosamine, galactose and specific

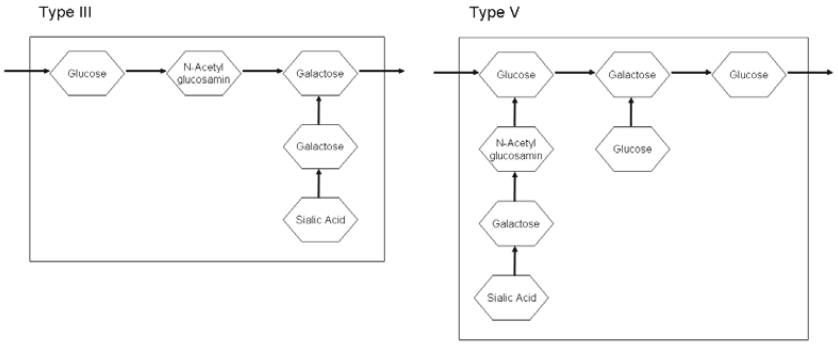
for GBS glucitol (Madoff et al., 2006; Pritchard et al., 1981). These sugars form a tetra-antennary structure protruding from the cell surface (Michon et al., 1987). Group B-specific antibodies have shown opsonizing activity on bovine strains which often possess low levels of CPS. On human strains which usually are highly encapsulated group B-specific antibodies are not able to bind to the group B antigen. (Madoff et al., 2006; Marques et al., 1994).

1.2.2 Capsular polysaccharides

GBS is usually encapsulated by a polysaccharide capsule, which is a major virulence determinant. Ten different of these capsular polysaccharides (CPS), designated Ia, Ib and II to IX, have so far been identified. By serotyping about 5-10% of strains are found to be non-typable, although the genetic information for CPS formation is almost always present when the strains are typed by PCR (Kong et al., 2002; Slotved et al., 2003). The different polysaccharides occur at different frequencies, with CPS types III and V as the most common followed by Ia, II and recently IV (Diedrick et al., 2010; Madoff et al., 2006). There are, however, geographical variations in the prevalence of CPS types, e.g. in Japanese women types VI and VIII are predominant (Lachenauer et al., 1999). Also certain CPS types are found more often in certain groups of patients, most notably, type III which causes up to 50% of newborn infections (Diedrick et al., 2010; Edwards and Nizet, 2011).

The polysaccharides are built up of subunits of oligosaccharides with a backbone structure and side chains as illustrated in Figure 2. The oligosaccharide subunits are composed of four to seven monosaccharides depending on the serotype. Glucose, galactose, N-acetylglucosamine, rhamnose and sialic acid are used in varying amounts (Madoff et al., 2006). The subunits are repeated, usually 100 or more times (Rubens et al., 1987). The composition and architecture of backbone and side chain varies, resulting in immunological distinct CPS types. The polysaccharide capsule interferes with the deposition of complement components on the bacterial surface. Sialic acid is present in all nine types that are investigated while no data are available for the recently described CPS type IX (Slotved et al., 2007). Sialic acid holds the terminal position of the side chain and is a pathogenicity factor in itself because it inhibits the activation of the alternative complement pathway (Edwards et al., 1982; Madoff et al., 2006; Marques et al., 1992; Wessels et al., 1989). Antibodies against CPS can mediate type specific protective immunity.

Figure 2: Illustration of the structure of the oligosaccharide subunits of the GBS capsular polysaccharides. The subunits consist of a backbone with side chains. The subunits are usually repeated 100 times and more. Type III with a subunit with five monocaccharides, type V with seven are shown here.



1.2.3 Surface proteins

GBS express a variety of surface proteins, some of which are present in every strain such as the Sip or the FbsA protein discussed below. Other proteins are found in some but not all strains, and have been used for sero-subtyping purposes, most importantly the alpha-like protein (Alp) group. A comprehensive overview of GBS surface proteins was published in 2005 (Lindahl et al., 2005).

1.2.3.1 Alps and other strain variable surface proteins

Of the GBS surface proteins, the strain variable alpha-like proteins have been most extensively studied. The Alps include six known proteins designated Cα, Alp1, Alp2, Alp3, Alp4 and R4. One of them is found in almost all GBS strains but only very rarely more than one Alp is present. The nomenclature is incoherent. The prototype protein of the group is Cα (Madoff et al., 2006). Strains which are positive for the Cα protein usually harbor the Cβ protein as well which is discussed below. Alp1 is closely related to Cα but not identical and is termed Epsilon by some authors (Creti et al., 2004; Puopolo and Madoff, 2003). Strains with Alp1 usually do not possess the Cβ protein. Alp4, originally detected in a bovine GBS strain, occurs infrequently and has to our knowledge never been encountered in human strains. The R4 protein was shown to be identical to the Rib protein, both designations are still in use (Bevanger et al., 1995; Smith et al., 2004). The genes for all of these six Alps have been sequenced, except for Alp4 for which only a partial sequence is available. The genes are named *bca* for the Cα protein, *alp1* (or *epsilon*) for Alp1, *alp2* (Alp2), *alp3* (Alp3), *alp4*

(Alp4) and *rib* (R4). When tested immunologically some strains do not react with any of the Alp antisera even if the genetic information for the protein is present in the genome. One of these genes has been found in almost every GBS strain tested. The proportion of immunologically non-typeable strains may depend on the strain collection, antisera used and the immunological methods. The Alps are usually associated with certain capsular types, e.g. CPS type Ia is usually found together with Alp1, type III with R4 and type V with Alp3 (Lindahl et al., 2005).

All Alps are constructed in a similar manner; they consist of a C-terminal end containing the LPXTG motif, typical for the cell wall anchoring part of surface-anchored proteins in gram-positive bacteria. Towards the N-terminal this is followed by a variable region and then long tandem repeats of different length and repeat number. The *bca* gene encoding Cα found in the fully sequenced reference strain A909 has nine completely identical repeats of 246 nucleotides (Michel et al., 1992). These large repeats result in Western-Blot patterns with a typical ladder formation (Madoff et al., 2006). It was shown in Cα strains isolated from mothers and their newborn children that the strains from children had fewer repeats than the strains from the mothers. The low repeat mutants were less efficiently opsonized for phagocytic killing than the strains from mothers (Madoff et al., 1996; Madoff et al., 2006). The N-terminal end is distinct for each protein, except for Alp2 and Alp3, and it often harbors a specific antigenic site. Alps are built of interchangeable units in a mosaic fashion (Lachenauer et al., 2000). For instance, R4 and Alp3 have nearly identical repeat units, Cα and Alp1 have identical repeat units and Alp2 and Alp3 have identical N-termini (Kvam et al., 2011). These similarities can probably explain the immunological cross-reactivity observed. Cross-reactivity due to the mosaic structure however, makes the use of these proteins for subtyping challenging. This is especially a problem for the Alp3 protein which is thought to have no protein specific antigenic sites of its own, but reacts with antisera against Alp2 and R4. Typing of this protein by immunological methods is therefore difficult. Also Alp1 is difficult to distinguish from the closely related Cα by serotyping. The existence of Alp1 was suspected through immunological studies and confirmed by sequencing in 1994 (Brady et al., 1988; Madoff et al., 2006). Studies published before the recognition of Alp1 would have reported strains containing Alp1 as Cα without Cβ. The erroneous classification was due to strong immunological cross-reactivity. In a recent study an Alp1 specific antigenic site has been described (Kvam et al., 2011).

Proteins of the Alp-family may also be found in other streptococci. The R28 protein in *Streptococcus pyogenes* is nearly identical to the Alp3 protein of GBS. It might have arisen in

GBS and may later have been acquired by *S. pyogenes* by horizontal gene transfer (Stalhammar-Carlemalm et al., 1999). In a *Streptococcus dysgalactiae* subsp. *equisimilis* strain a chimeric protein of R4 and Alp2 was demonstrated (Creti et al., 2007).

Other strain variable surface proteins are the Cβ protein mentioned above, the R3 and the Z proteins. Cβ usually accompanies the Cα protein; often in CPS type Ib strains (Madoff et al., 2006). It was first recognized as part of the C protein of which a trypsin resistant component was found to be Cα and a trypsin sensitive component was Cβ (Bevanger and Maeland, 1979; Wilkinson and Eagon, 1971). Cβ is a 130-kDa membrane bound protein. The sequence of the Cβ gene *bac* is known, among others from the fully sequenced strain A909. It does not have large repeats as those found in alpha-like proteins. Cβ has a domain in its N-terminal half with high affinity to human IgA binding it in a non-immunological fashion. Immunization of mice with this protein protected their neonatal pups from GBS infection (Madoff et al., 1992). It has therefore been proposed as a conjugate component for a possible CPS/Cβ conjugate vaccine (Madoff et al., 1994). The R3 protein was described in 1972 as one of four R proteins found in streptococci (Wilkinson, 1972). Like the Alps described above, it is also a trypsin resistant and ladder forming protein. It is usually found in combination with an alpha-like protein. The sequence of the gene encoding R3 is not known. In most strain collections a low prevalence of R3 was found (< 10%) but in two Zimbabwean collections it was found in more than 20 % of all strains and in over 75% of serotype V strains (Mavenyengwa et al., 2008; Moyo et al., 2002). R3 may therefore be more important in certain geographical areas than in others. The Z protein was recently described and usually occurred in strains which also carried the R3 protein (Mavenyengwa et al., 2009).

1.2.3.2 Other surface proteins

Several other surface proteins have been identified and tested for their involvement in GBS virulence. The *scpB-lmb* composite transposon codes for C5a-peptidase and laminin-binding protein. Homologues of this transposon were found in the genomes of *S. pyogenes* and *S. dysgalactiae* subsp. *equisimilis*. The *scpB-lmb* genes are separated by a spacer region of 164 bp. Alternatively an insertion element named IS*1548* may be inserted into this intergenic spacer or as a third possibility another element named GBSi1 might be found. Interestingly these two inserted elements were shown to be associated with two CPS type III clonal complexes (CC, as determined by multi-locus sequence typing discussed below). IS*1548* was always found in CC19 and GBSi1 always in CC17 (Granlund et al., 2001; Hery-Arnaud et al., 2005). The C5a peptidase encoded by *scpB* cleaves the complement component

C5a which is a potent chemotaxin for polymorphonuclear leukocytes (Beckmann et al., 2002). Further the peptidase mediates binding of GBS to human immobilized fibronectin and has been shown to be involved in the invasion of epithelial cells by GBS (Cheng et al., 2002). The lipoprotein encoded by *lmb* mediates binding of GBS to human laminin and thereby to epithelial cells (Bröker and Spellerberg, 2004). The *scpB* and *lmb* genes seem to be harbored by all human GBS, but is rarely found in bovine strains (Dmitriev et al., 2004).

The FbsA protein is a surface exposed protein which binds to human fibrinogen and is therefore involved in the adhesion of GBS to human cells (Schubert et al., 2002). It has typical features of a surface located protein with a signal peptide sequence at the N-terminus, a cell wall spanning region and the typical anchoring motif LPXTG. Its most striking feature however is the middle part consisting of tandem repeats of 48 bp which is highly variable in number (Schubert et al., 2002). The number of repeats has been shown to have effects on the binding efficiency of FbsA to fibrinogen. Those strains with lower repeat counts had the highest efficiency in these experiments. Most of these strains belong to the clonal complex 17 (Rosenau et al., 2007).

The *bibA* gene encoding the BibA protein has been found in all GBS strains tested. The protein harbors the LPXTG motif but surface exposure is only found in about half of the strains tested. Surface exposure of the protein was associated with protection in mice immunized with BibA. The protein confers resistance to phagocytic killing and confers adhesion to host cells (Santi et al., 2007). The protein has been found in four allelic variants which are associated with specific capsular types and MLST clonal complexes (Lamy et al., 2006; Santi et al., 2009). One of the four variants is strongly associated with the CC17 and a PCR assay has been developed based on *bib*A for presumptive identification of the CC (Lamy et al., 2006).

The *sip* gene, encoding the surface immunogenic protein, is found in virtually all GBS strains. Its sequence is highly conserved (Brodeur et al., 2000). It may therefore be used for the detection of GBS by PCR (Bergh et al., 2004; Bergseng et al., 2007). Since immunization of mice with the Sip protein produced protective antibodies against challenge with several CPS types it is considered a vaccine candidate (Martin et al., 2002). The exact function of this protein is still unknown.

The serine-rich repeat protein Srr-1 is secreted extracellular and transported to the cell surface where it is heavily glycosylated. It was reported to promote colonization by enhancing adhesion, also an enhanced penetration of the blood-brain barrier by GBS in mice was reported (Sheen et al., 2011; van Sorge et al., 2009). Homologs of the protein exist in several

streptococcal species. A second serine-rich repeat protein Srr-2 has been described and seems to be associated with the important CPS III, sequence type 17 (Seifert et al., 2006).

Hyaluronate lyase is encoded by *hylB* and is thought to be associated with cell invasion. The insertion element IS*1548* mentioned above can sometimes be found in several copies in the genome and may be inserted into the *hylB* gene. These strains, many of which are invasive, can not generate hyaluronate lyase and its importance as a virulence factor is therefore doubtful since strains with or without the lyase seem to be equally virulent (Sukhnanand et al., 2005; Yildirim et al., 2002).

1.2.4 Pili

Pili on the surface of bacteria are promoting adherence to epithelial cells. Other functions may be to facilitate the formation of microcolonies and biofilms and to promote transepithelial migration (Margarit et al., 2009). In GBS, pili were found in 2005 by a reverse genetics approach (Dramsi et al., 2006; Lauer et al., 2005) which subsequently led to the discovery of pili in *S. pyogenes* and *S. pneumoniae* (Barocchi et al., 2006; Mora et al., 2005). Further studies in GBS showed three types of pili, termed 1, 2a and 2b. The encoding genes for type 1 and 2a/2b are located at two different locations in the genome (Telford et al., 2006). At least one of the three has been found in virtually all GBS strains tested and they often appear in combinations. Certain pili or combinations seem to be associated with certain serotypes. They have evoked protective immunity in mice and are therefore considered as new candidates for a GBS vaccine (Margarit et al., 2009).

1.2.5 Other virulence factors

Several other virulence factors of which most are secreted have been described in GBS. The CAMP factor used for presumptive identification of GBS has been shown to act as a co-hemolysin together with the *Staphylococcus aureus* hemolysin sphingomyelinase. They produce an enhanced hemolysis when cultured together on blood agar plates. CAMP factor has the ability to produce pores in target cells. Its pathogenicity has, however, been questioned (Hensler et al., 2008).

A cell-surface-associated protein (CspA) has been identified as an extracellular surface associated protease. The protein can cleave human fibrinogen and selected chemotaxins (Bryan and Shelver, 2009; Harris et al., 2003). CspA has been shown to be important for GBS to be fully virulent.

The hemolysin responsible for GBS β-hemolytic activity is β-hemolysin/cytolysin encoded by the *cyl* operon. It is a pore forming cytolysin. It is not related to the streptolysins in *S. pyogenes* which are major virulence factors of that species. The occasional appearance of non-hemolytic clinical isolates suggests that the hemolysin is not vital for the pathogenicity of GBS. It has not enhanced GBS virulence in rats (Weiser and Rubens, 1987). More recent studies found cytotoxic activity in the neuronal tissue of rats which might involve the protein in the pathogenesis of meningitis (Reiß et al., 2011). Non-hemolytic strains seem to have a insertion sequence in their *cyl* operon disturbing hemolysin production (Spellerberg et al., 1999).

1.3 Genetics of GBS

In 1995 the genome of a *Haemophilus influenzae* strain was published (Fleischmann et al., 1995), the first free-living organism to have its entire genome sequenced. This was possible because of advances in sequencing technology, especially the chain termination technology (Sanger et al., 1977) and the sophistication of this by fluorescence detection of DNA fragments (Smith et al., 1986). This technology was afterwards used to sequence the genome of other bacterial species. For the Streptococcus genus two genomes each of *Streptococcus pyogenes* and *Streptococcus pneumoniae* were published in 2001 and 2002. Later in 2002 two whole genome sequences of GBS were published by two independent groups at the Pasteur institute (Glaser et al., 2002) and the TIGR institute (Tettelin et al., 2002). Comparisons of the genomes of the three species showed that most of the proteins found in the GBS genome had orthologs in at least one of the two other species. The chromosomal order is highly conserved between GBS and *S. pyogenes*, underscoring the relatedness of these two species (Glaser et al., 2002).

The Pasteur institute in Paris sequenced the strain NEM316 of CPS type III, Alp2 surface protein and multi-locus sequence type (MLST) 23. Strain 2603V/R (CPS type V, protein R4, MLST110) was sequenced by the TIGR institute in Maryland, United States. These genomes were sequenced by the shotgun procedure and afterwards assembled and annotated. Both are somewhat atypical representatives of their serotypes, both with regard to surface proteins and sequence types (as determined by multi-locus sequence typing discussed below). NEM316 (ATCC12403) is a strain from before the Second World War and was given to Lancefield by Colebrook according to the ATCC catalogue (LCG_Standards, 2011). It is often referred to as an invasive neonatal strain, but this has been questioned (Sørensen et al.,

2010). Its surface protein is Alp2 instead of the expected R4 and the sequence type is ST23, whereas a typical pathogenic type III strain would be expected to be ST17 or ST19. Further it is lactose fermenting, which is more typical for bovine strains. The strain 2603V/R displays the R4 protein instead of the more common Alp3 and it is of ST110 (clonal complex 19) and not ST1 which would have been typical for a CPS type V strain. Capsule switching might be responsible for the finding of CPS type V in 2603V/R (Davies et al., 2004). Another fully assembled genome is published for strain A909 (CPS type Ia, proteins Cα, Cβ, ST7) (Tettelin et al., 2005), also in this strain the presence of Alp1 and the absence of Cβ would be more typical for a Ia strain. Five additional strains were shotgun sequenced but not fully assembled (strain 515 (Ia, epsilon, ST23), H36B (Ib, Cα, Cβ, ST 6), 18RS21 (II, ST19), COH1 (III, ST17) and CJB111 (V, ST1) (Tettelin et al., 2005). In 2010 an additional shotgun sequence became available as part of the human microbiome project (ATCC13813, Ic, GeneBank accession number: AEQQ00000000) and in 2011 the genome of a strain from bovine mastitis assembled to only eight contigs was published (Richards et al., 2011).

GBS has a circular genome of around 2.2 mill bp with a low G+C content of ca. 35%, typical for streptococci. The bovine strain sequenced by Richards et al. had a considerably larger genome of 2.45 mill bp mainly due to insertion sequences (Richards et al., 2011). The GBS genome contains about 2100 genes of which two-thirds have assigned biological roles (Tettelin et al., 2002). While about 55% of the genes in the genome of strain NEM316 have orthologes in the genome of *S. pyogenes*, most of the genes that do not have orthologes, are clustered in 14 genomic islands initially described as putative pathogenicity islands (Glaser et al., 2002). Further studies confirmed this assumption for four of these (Herbert et al., 2005).

Bacterial genomes have been divided into a conserved core genome and a more variable accessory genome. The core genome consists of genes encountered in all strains of the species and the accessory genome is the sum of all genes not present in all strains in sequenced strains of a species. Together they represent the pan-genome of the species (Tettelin et al., 2005). In a comparison between streptococcal genomes, the genome of GBS has been found to have a larger pan-genome than the closely related *S. pyogenes* possibly reflecting the adaption to a broader habitat by GBS (Lefebure and Stanhope, 2007). On the other hand the core genome of GBS seems to be better conserved than that of *S. pyogenes* with 18% and 37% putative recombinant genes, respectively. Because of the broader habitat the size of the GBS pan-genome is thought to be less well estimated by the sequences of eight genomes, a statement that is underscored by the bigger size of the recently sequenced bovine strain with 183 genes specific to this strain (Richards et al., 2011).

1.4 Epidemiology of GBS

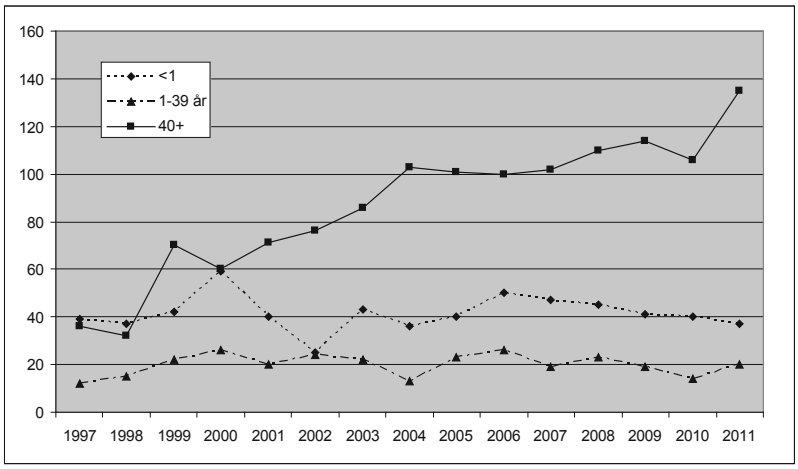
GBS is regularly found in humans as a colonizing organism without causing symptoms. The main habitat of the bacterium is the gastrointestinal tract; this location leads to a colonization of the female genitourinary tract (Edwards and Nizet, 2011). GBS from women in childbearing age has been recovered at variable frequencies, but with recto-vaginal samples and optimized culture techniques frequencies of 21-35% have often been reported in newer studies (Bergseng et al., 2007; Madzivhandila et al., 2011; Mavenyengwa et al., 2010; Van Dyke et al., 2009). In a recent review of colonization rates in Europe a range from 6.5% (Turkey) to 36% (Denmark) was noted (Barcaite et al., 2008). Colonization with GBS in pregnant women is intermittent in a considerable number of women. Late antenatal cultures performed no longer than 5 weeks before delivery are therefore considered fairly accurate in predicting the carrier status at delivery (Yancey et al., 1996). GBS can also be found in the urethra and is an unusual cause of urinary tract infection. Other locations such as the oro-pharynx and upper airways have been reported, however at much lower frequencies. During delivery, the child of a colonized mother can become infected. Colonization in late pregnancy is therefore a risk factor for newborn disease, and screening for GBS colonization is performed in several countries. To determine the GBS colonization status it is recommended to culture swabs collected from the lower vagina and rectum. The use of selective enrichment broth is recommended and improves the chance of laboratory detection of GBS substantially (Verani et al., 2010).

1.4.1 Infection and disease in humans

GBS is a colonizing organism in humans but can occur as an opportunistic pathogen. Three patient groups can be separated: nonpregnant adults, newborn children and pregnant women. About two-thirds of all invasive GBS cases in the USA in 2001 were encountered among nonpregnant adults and the frequency of invasive diseases in this patient group seems to increase further (Skoff et al., 2009), an observation also made in Norway (Figure 3). Most of the patients in this group do have underlying diseases such as diabetes or malignancies (Farley, 2001). The risk of GBS disease is also increasing with age. While septicemia without identifiable focus is observed regularly in nonpregnant adults, syndromes such as skin and/or soft tissue infection, pneumonia and septic arthritis are also common (Schuchat et al., 2006;

Skoff et al., 2009). Cases of necrotizing fasciitis are observed infrequently (Sendi et al., 2008).

Figure 3: Number of cases of invasive group B streptococcal disease for three age groups as notified to the Norwegian Surveillance System for Communicable Diseases (www.MSIS.no), years 1997-2011



Among newborn children, GBS is a leading cause of invasive bacterial disease (Schuchat et al., 2006). The neonatal patients are classified into two groups: those who become ill on days 0-6 of their life, referred to as early onset disease (EOD) and those affected on days 7-90 after birth, referred to as late onset disease (LOD). Colonization of the newborn child is a prerequisite of EOD. Vertical transmission of GBS from colonized mothers to their newborns occurs in about 50 % of births (Edwards and Nizet, 2011). Transmission might occur by the ascending route into the uterus, through translocation through intact membranes, through ruptured membranes, or by contamination during passage through the birth canal. There is an increased risk of colonization of the newborn if the mother is heavily colonized (Ferrieri et al., 1977; Regan et al., 1996). Overall only few neonates develop invasive GBS disease; usually less than one per 1000 life births in industrialized countries, however the incidence may be higher in developing countries (Madhi et al., 2003). Most cases of invasive disease occur in adults but the incidence is considerably higher in newborns. In Norway the annual incidence rate for neonatal disease is 0.70/1000 for the period 2001-2010 (Folkehelseinstituttet, 2011).

Few newborns, usually between less than 0.5 to 1/1000 live births, will develop EOD. The annual incidence rate in Norway for EOD for the years 2005-2010 is 0.42 (own data). In the USA a prevention strategy has succeeded in reducing the incidence from around 2/1000

live births to 0.34 in 2003-2005 (Phares et al., 2008). When considering a maternal colonization rate of 25-33% and a newborn colonization rate of ca. 50%, about 12.5-17.5% of newborns become carriers of GBS at birth and are at risk for EOD. Most fatal cases in newborns occur in the EOD group. The case-fatality rate is reported to be 20% in infants before 33 weeks of gestation and about 2-3% among full-term infants (Verani et al., 2010). EOD will typically develop as septicemia, pneumonia, meningitis or other serious syndromes. About 85% of EOD cases occur within 24 hours of birth. Most premature infants tend to be in this group while babies with onset after 24 hours tend to be born at term (Edwards and Nizet, 2011).

About 20 to 40 % of neonatal cases have been classified as LOD but with successful EOD prevention programs the proportion of LOD will necessarily increase. In the USA the proportion has been reported to be around 50% (Phares et al., 2008). Among babies who develop LOD 65% of cases may develop septicemia without identified focus (Edwards and Nizet, 2011). Meningitis is a common presentation and found in 25% of cases. About 50% of these cases suffer from long-term neurodevelopmental sequelae (Bedford et al., 2001). Risk factors for LOD are less well defined than for EOD. GBS is acquired perinatally, from community sources or nosocomially. Prematurity is the best recognized risk factor for LOD, however in term infants, obvious risk factors can often not be identified (Edwards and Nizet, 2011; Jordan et al., 2008; Lin et al., 2003). A minority of cases might be associated to GBS in breast milk (Gagneur et al., 2009; Kotiw et al., 2003).

Pregnant women may develop GBS related diseases during their pregnancy, childbirth or the postpartum period (Schuchat et al., 2006). Urinary tract infection caused by GBS is sometimes observed during pregnancy. More serious conditions such as chorioamnionitis or bacteremia may occur. Studies found that such diseases accounted for 11% (Zangwill et al., 1992) and 6.3% (Schrag et al., 2000) of all invasive GBS cases. Further, infections of the mother may lead to premature delivery and low birth weight infants or late abortions (Daugaard et al., 1988). Also GBS can be found after stillbirth and late abortion in autopsies and may be considered a causative agent (Gibbs et al., 2004; McClure et al., 2010). GBS may be found in breast milk with mastitis symptoms in about 21% of mastitis cases and in 10% of controls (Kvist et al., 2008).

1.4.2 Prevention of GBS disease

Two main strategies for prevention of human GBS disease are focused upon in the research community today: intrapartum antibiotic prophylaxis (IAP) and development of a

vaccine against GBS. It is well documented that vertical transmission of the agent in newborns can be reduced by administration of intrapartum prophylactic antibiotics to the mother (Verani et al., 2010). This strategy has succeeded in reducing the incidence of EOD in the USA (Phares et al., 2008). Vaccination would have the potential to prevent LOD and adult disease as well. Several candidate vaccines have been tested successfully and clinical trials are underway (Heath, 2011).

Prevention of EOD in newborns is possible when antibiotics are given to the mother during delivery, i.e. intrapartum antibiotic prophylaxis. Selection of pregnant women for IAP follows two main strategies; either a risk-based approach or an approach based on screening for recto-vaginal GBS colonization in pregnancy week 35-37. The risk-based strategy includes a set of criteria known to increase the risk of EOD in newborns. The following risk factors have been defined: GBS colonization, GBS bacteriuria as a marker of heavy colonization or GBS urinary tract infections of the mother during pregnancy, preterm birth before 37^{th} week or low birth weight infants, prolonged rupture of membranes >18 hours, temperature > 38ºC, and a previous infant with GBS disease (Verani et al., 2010). If any of the risk factors is present the woman should receive IAP. The drug of choice in this setting is intravenous penicillin, while clindamycin or erythromycin is used if penicillin is contraindicated. IAP also reduces the frequency of invasive GBS disease in mothers (Phares et al., 2008).

The screening- or culture-based strategy includes screening of all pregnant women for GBS colonization in pregnancy week 35-37. All colonized women should then receive IAP. This approach has been adopted in the USA through the 1990s with U.S. American national guidelines describing these measures first released in 1996 and updated in 2002 and 2010 (Verani et al., 2010). This strategy has resulted in a considerable reduction in cases of EOD from 1.7 cases per 1000 live births in the early 1990s to 0.5 in 1999 and 0.34-0.37 cases in 2003-2008 (Jordan et al., 2008; Verani et al., 2010). Most European countries chose to follow the risk based strategy in the 1990s because of a generally lower incidence of EOD. In recent years however the apparent success of the screening based strategy has caused countries like France and Germany to adopt American guidelines. Other countries such as Norway and the UK are following the risk based approach (Hordnes et al., 2010).

1.4.2.1 Vaccines

IAP prevents only EOD while vaccination is expected to prevent LOD as well. D (Heath, 2011). A vaccine could be administered to adolescent females or late in pregnancy. Also adults at risk (>65 years, diabetics) could be selected for targeted immunization.

Many antigens of GBS have been proposed as candidates for a vaccine. Most efforts have concentrated on using CPS as immunoprophylactic antigens. The CPS type most frequently encountered in neonatal disease is type III followed by types Ia, Ib, II and V. Together they are responsible for about 96% of neonatal and 88% of adult cases in the USA (Phares et al., 2008). A CPS vaccine should therefore include these antigens. Candidate vaccines using CPS alone have shown poor immunogenicity. In newer candidate vaccines CPS are therefore conjugated to protein carriers. Several proteins have been tested as conjugates, e.g. tetanus toxoid or CRM197, a non-toxic diphtheroidal protein. GBS surface proteins have been used successfully as conjugates with CPS in mice and have an obvious attraction as they confer immunity on their own (Heath, 2011; Madoff et al., 1994). Other targets than CPS for a GBS vaccine have been proposed. Several surface proteins of GBS have been shown protective in animal studies, e.g. Sip (Brodeur et al., 2000) and C5a peptidase (Santillan et al., 2008). Both proteins are present in all GBS strains and would have obvious advantages in design over vaccines combined of several CPS types. The recent discovery of pili has been a result of genome mining and has added another interesting group of antigens to the list of GBS vaccine candidates (Margarit et al., 2009).

1.4.3 Infection in animals

GBS infection has been reported in several different animal species such as bovines, dog, cat, goat, elephant, fish, crocodile and frog (Bishop et al., 2007). In veterinary literature mastitis is studied mainly in domestic cattle, but other milk producing animals such as camels or sheep can develop GBS mastitis (Linage and Gonzalo, 2008; Tibary et al., 2006). In other animal species GBS may infect different organ systems resulting in outbreaks of meningoencephalitis and septicemia in fish farms or outbreaks of necrotizing fasciitis on crocodile farms (Bishop et al., 2007; Pereira et al., 2010).

1.4.3.1 Infection in cattle

In cattle, GBS is an obligate pathogen of the udder leading to acute mastitis or to subclinical mastitis (McDonald, 1977). Other sites of infections in cattle are virtually unknown in veterinary literature (Zadoks et al., 2011). Acute mastitis with fever and

inflammation of one or several quarters of the udder is usually readily diagnosed and treated. Subclinical infection is at least equally common and does not have a high self-cure rate (Keefe, 1997). It leads to an inflammation of the milk ducts and gradual scaring with decreasing milk production. Animals with subclinical disease are thought to be the reservoir for maintaining outbreaks in herds by going unnoticed and spreading the bacterium to other individuals. In a Danish Study the udder was artificially infected with GBS strains obtained from humans or bovines. Results suggested that the strain origin might have an influence on the outcome. If the strain was a human colonizing strain it lead to acute mastitis with a tendency towards self-cure. If the strain was of bovine origin the acute disease tended to be milder, however self-cure was unusual (Jensen, 1982). Studies analyzing outbreaks of GBS identify usually a single strain responsible for an outbreak in a herd, while different herds have different strains (Barkema et al., 2009; Duarte et al., 2004; Zadoks et al., 2011).

Mastitis makes a serious economical impact on milk production. The annual costs of mastitis were estimated to be around 245 million Norwegian kroner in 2000. This is a result of the combined costs of diagnostics and treatment, discarded milk in the disease period, reduced milk production afterwards and reduced prices because of elevated cell counts in the milk (Østerås and Lystad, 2001). Previously, GBS was a major cause of mastitis in dairy cows, especially in the pre-antibiotic era (Keefe, 1997). E.g. for Danish herds in the 1950s a prevalence of GBS infected herds of 20-30% was observed (Jensen, 1980). Due to the high prevalence eradication programs were instituted to control GBS mastitis. Measures included education of farmers and infection control measures in stables. When GBS was found in a herd, treatment of infected animals was instituted. In more severely affected herds all animals were screened, subclinical infections were treated and finally animals which remained infected after repeated treatment attempts were culled. Surveillance of mastitis is usually carried out by screening the cell count in bulk milk. High cell counts indicate the presence of leukocytes and inflammation and should trigger follow-up measures (Keefe, 1997; McDonald, 1977). The efforts to eradicate bovine GBS mastitis succeeded in reducing the incidence through the last 30-40 years, and mastitis by GBS was rarely encountered in Scandinavia since the 1980s, e.g. Denmark had a herd prevalence of <2% through the 1980s and 1990s (Agger et al., 1994). This success led to the abandonment of screening of bulk milk for GBS in Norway in 1996.

During the last ten years a reemergence of GBS mastitis has been observed in several Scandinavian countries, and in 2008 close to 6% of Danish herds were found positive (Barkema et al., 2009; Katholm and Rattenborg, 2009; Zadoks et al., 2011). Newer

developments towards free stalls, bigger herds, milking robots and organic farming have been suggested as contributing factors (Persson and Landin, 2009).

Given the close relationship between humans and cattle through history, the idea of a mutual exchange of this common pathogen is obvious. The cohabitation of bovines and humans on farms has given ample opportunity for GBS transmission between humans and bovines in many situations including the ingestion of unpasteurized milk as a possible source for transmission to humans. It is likely that there was a common ancestor. One of the most important epidemiological questions of today is if an exchange of strains between human and cattle still occurs or if human and bovine strains are distinct entities. The possibility of a more easy clearance of human strains from infected udders may be an indication of separate entities (Jensen, 1982). Several studies based on phenotypic markers concluded that GBS isolated from bovines and humans are separate ecovars. This was based on tests such as hemolysis, pigmentation and fermentation of salicin and lactose (Butter and de Moor, 1967; Finch and Martin, 1984; Jelínkoá, 1977). These older reports have been supported in newer studies using molecular methods. Most of these more recent studies argue that bovine GBS are distinct from human GBS (Bohnsack et al., 2004; Martinez et al., 2000; Sukhnanand et al., 2005; Sørensen et al., 2010). Also the first published genome of a bovine strain indicates that bovine and human GBS represent distinctive lineages (Richards et al., 2011). Some evidence exists for bovine ancestry of the human serotype III, ST17 strains often found in neonatal disease. It was shown to be related to bovine ST61 strains (Bisharat et al., 2004; Hery-Arnaud et al., 2007).

1.5 Laboratory detection of GBS

GBS grows readily on blood agar, in broth or blood culture vials. The detection of the bacterium in samples from the vagina and/or rectum can be more challenging. The use of enrichment broths such as Todd-Hewitt broth, with antimicrobial agents suppressing gram-negative flora is therefore recommended for detection of colonization (Spellerberg and Brandt, 2011; Verani et al., 2010). After enrichment in broth, subculture on blood agar plates is used for further processing. The CAMP-test or latex agglutination tests reacting with the group B antigen will usually lead to the identification of GBS (Spellerberg et al., 1999). Lately chromogenic media have been introduced to alleviate the detection of GBS in multibacterial samples. A color change in the presence of colonies of GBS facilitates detection. Identification of non-hemolytic strains however may be challenging (Verani et al., 2010).

Gene-based test such as probes or PCR have also been used to identify GBS. Real-time PCR assays allow the rapid detection of GBS with high sensitivity. Several GBS genes have been used as targets such as the *cfb* gene coding for the CAMP factor (Ke et al., 2000), the *sip* gene (Bergh et al., 2004; Bergseng et al., 2007) or the *pts*1 gene (Uhl et al., 2005). Although the sensitivity of such PCRs can be excellent even without previous enrichment (Bergseng et al., 2007), American guidelines recommend the use of enrichment to maximize sensitivity (Verani et al., 2010).

For screening purposes the use of enrichment broth and subsequent culture on agar media results in a sample turnaround time of at least two days. Fast gene-based tests without enrichment would theoretically allow screening of pregnant women in labor and give a more correct diagnosis of colonization than screening in week 35-37. Several studies have investigated the performance of gene-based tests without enrichment. Commercial PCR tests designed for this purpose are available, e.g. the BD GeneOhm system (Becton Dickinson, Trondheim, Norway) and the GeneXpert system (Cepheid Europe, Maurens-Scopont, France), both using the *cfb* gene. With rapid processing, these systems may be able to detect the GBS colonization status at delivery and thereby replace screening in weeks 35-37 by antepartum or intrapartum screening. The tests have shown good sensitivity and specificity in some but not all studies as summarized by Verani (Verani et al., 2010). Because of their variable performance, the problem of turnaround time under obstetric routine conditions, eventual delays in administration of antibiotics, costs and other unsolved problems, gene-based tests at delivery are for the present regarded as supplemental to screening by culture and risk-based approaches (Spellerberg and Brandt, 2011; Verani et al., 2010).

In cattle with acute mastitis milk from infected quarters of the udder should be cultured and GBS or other bacteria causing mastitis can be identified by standard microbiological methods. In subclinical mastitis individual or composite samples of all four quarters should be cultured. The demonstration of GBS in bulk milk indicates infected individuals in a herd and should result in follow up sampling (Keefe, 1997). Lately multiplex-PCR systems have become commercially available which are able to diagnose several mastitis pathogens in one assay, among them GBS (Koskinen et al., 2009).

1.6 Considerations regarding the typing of bacteria

An evaluation of the relatedness of bacterial isolates may be necessary in several settings. For the microbiological routine laboratory or in a reference laboratory the need for

typing of bacteria will often arise when two or several samples are suspected to be epidemiologically connected, e.g. in nosocomial or food-borne outbreaks. Another setting might be the epidemiological surveillance of an infectious disease over time to follow disease trends and designing possible ways of infection control. As part of a surveillance approach, it is highly desirable to store typing results from outbreaks for comparison with future outbreaks or other research. Typing methods which produce numerical, unambiguous results will alleviate the exchange and comparison of results in scientific networks, e.g. through databases accessible via the internet (van Belkum et al., 2007). Another possible application of typing methods is the comparison of strains of a bacterial species in a single patient to differentiate pathogenic from nonpathogenic or endogenic from exogenic strains. Finally, typing systems may be used to determine the intraspecies population structure and lead to phylogenetic hypotheses (Feil, 2004; Smith et al., 1993; van Belkum et al., 2007). Phylogenetic analysis needs a careful selection of markers. Slowly evolving markers such as ribosomal or housekeeping genes are thought to represent a better estimate of the phylogeny of species than more quickly evolving genes of surface proteins or repeated sequences (Feil, 2004). In research established methods or dedicated typing tools might be designed for epidemiological comparisons, phylogenetic studies, study of virulence markers or other characteristics. The methods used in the different situations sketched above will group the isolates according to the discriminatory power of the method. The discriminatory power refers to the ability of a method to assign a different type to two unrelated strains sampled randomly from the population of a given species (Foxman et al., 2005; van Belkum et al., 2007).

In 2005 a publication which introduced the concept of a bacterial pan-genome used GBS as an example organism (Tettelin et al., 2005). In this concept, as mentioned previously, the pan-genome includes all genes ever found in strains of a given bacterial species. The pan-genome is divided into a core genome and a dispensable or accessory genome. The core genome consists of genes encountered in all strains of the species. These are housekeeping genes, ribosomal genes and other genes necessary for the basic functions of the cell (Feil, 2004). The accessory genome contains all genes ever found in one or more strains of the species, except for those of the core genome. The genes of the accessory genome usually encode for accessory properties such as pathogenicity. The accessory genome will become larger with each new sequenced strain. Based on the information from the eight strains in the article by Tettelin et al. mathematical modeling extrapolated that the pan-genome of GBS will increase by about 33 new genes (CI 22-42) with each new sequence becoming available. With this concept the additional genome will increase while the core-genome will slowly decrease,

as genes previously classified as part of the core genome may be found to be lacking in some strains as more genome sequences become available.

Typing methods are dependent on variations in the bacterial genome. The genetic events leading to such variation are mainly recombination and point mutation (Bessen, 2010). Recombination events are more frequent than mutations with rates of about nine to one (Feil et al., 2001). Recombination events change stretches of sequences while point mutations may affect only single nucleotides. Recombination is thought to be the main force of genetic change in several streptococci, among them GBS (Bessen, 2010; Brochet et al., 2008; Lefebure and Stanhope, 2007). Recombination and point mutations can occur throughout the whole genome but at different frequencies. Conserved parts of the genome usually encode proteins involved in processes which are essential for the functioning of the cell. Examples are housekeeping genes or ribosomal proteins. Successful mutations in these genes are uncommon since many mutational events may be lethal or leave the strain severely disadvantaged and exposed to negative selection (Didelot and Falush, 2007).

Conserved genes constitute the stable backbone of the genome. Genetic islands in a genome, on the other hand, are sequences which differ by a number of features from the backbone of the genome. Genetic islands are usually identified by a different G+C content than the core genome (Schmidt and Hensel, 2004). They are often thought to have been acquired and integrated into the genome by horizontal gene transfer. Genomic islands may or may not have retained features of movable genetic elements, but some, such as transposons, integrases and genomic material from bacteriophages will usually be detectable. The most integrated of these genetic islands may have lost all features of mobility and have become part of the core genome of the species. Islands which have gone through such adaptation may have contributed considerably to the fitness of the host organism (Hacker and Kaper, 2000).

Less integrated islands are usually the “hotspots” for variability. Mutations in these regions may potentially have influence on cell functions, but are usually not essential for the survival of the cell. Variability can also be favorable; it may lead to adaption to the host, or be involved in the control of gene expression. Pathogenicity islands can either be considered as genomic islands of their own, encoding virulence factors, or they may be part of larger genomic islands (Hacker and Kaper, 2000). The conserved and variable parts of the genome of bacteria described above have a different mutation rate, sometimes referred to as molecular clock speed. Examples of genetic events leading to different rates of mutation are negative selection as described above, horizontal gene transfer, homologous recombination or slipped stream mispairing in repeated sequences. In the context of typing, genes with low mutation

rates are preferable in the investigation of phylogenetic relationships and population dynamics. On the other hand genes with high mutation rates are well suited for outbreak investigation (Achtman, 2008; Foxman et al., 2005; van Belkum et al., 2001; van Belkum et al., 2007).

Repetitive sequences are often located upstream and downstream of genetic islands. Such repeated areas in the proximity of coding genes can affect the function of the genes (Hacker and Kaper, 2000). Especially short sequence repeats (SSR) of less then 9 bp per repeated unit can disturb the physical integrity of the gene by disrupting or modifying open reading frames (Janulczyk et al., 2010; van Belkum, 1999). Repeated sequences can also be part of functional DNA domains. These sequences tend to have longer repeat units. If they are diverse in their number of repeats, they are termed variable number of tandem repeats loci (VNTR). The tandemly repeated parts in the genes of proteins will often translate into helical or coiled structures on the cell surface (Fischetti, 2006). The alpha-like protein group of GBS is an example of this with rather long repeat units.

The need for subdivision of bacterial strains within a species has led to the development of many typing methods over time. Methods have been dependent on the technical possibilities of their time and many have become outdated and replaced by newer methods. Other typing methods have survived the test of time or have been adapted to new technical possibilities. An example for the latter is the typing of the capsular polysaccharides of GBS which was originally done by immunological methods, but is often done by gene-based methods today. The reproducibility of results achieved by a given typing method, both intra- and interlaboratory, should be determined since good reproducibility is a prerequisite for comparison of results between different test runs in the same laboratory or between different laboratories. For PFGE, an otherwise excellent method with high discriminatory power, this has been a central issue (van Belkum et al., 2007). Also resource limitations, such as costs, personnel competence and available equipment, may hamper the choice of an optimum method.

Historically phenotypic methods have been important for typing. Simple phenotypic methods such as colony appearance, biochemical differences and antibiotic susceptibility profiles might be used as initial or impromptu techniques. Immunological methods, phage typing or multi-locus enzyme electrophoresis (MLEE) have been used for typing of strains and can have high discriminatory power. Serotyping of Salmonella might be used as an example where immunologically based typing still is used as a standard method. More advanced phenotypic methods are often cumbersome and involve the maintenance of libraries

of antisera, standardization problems and problems of reproducibility. Such methods often depend on specialized personnel for interpretation. Raman spectroscopy is a new promising typing method which might be considered as a phenotypic method. It has the potential of rapid, high throughput typing of bacteria without extensive expert hands-on work (Willemse-Erix et al., 2009).

Methods based on the investigation of the genome of microbes have over the past 20 years in many settings replaced phenotypic methods and offer several advantages such as usually better resolution and in many cases better reproducibility. Such molecular methods are often technically advanced and may therefore be less convenient in resource limited settings which is often the case in developing countries. A wide variety of typing methods has been developed to investigate the diversity of bacterial genomes. Several methods use DNA hybridization where immobilized DNA is probed with selective DNA molecules. Array technology uses hybridization with a large number of targets and is able to screen all identified genes in prokaryotic species. Fragment based methods use restriction enzymes and analysis of the fragments by e.g. gel electrophoresis. Such methods might be combined with PCR-amplification steps such as in amplified restriction fragment length polymorphism (AFLP). PCR has been used in different typing methods, e.g. to amplify regions of known variability such as in multi-locus variable number of tandem repeats assays (MLVA). Methods based on sequencing of single or several loci in the genome are often used and have become the most important typing methods in some bacterial species. MLST, discussed below, is the main example of this (Pourcel and Vergnaud, 2011). A typing method targeting the gene for *Staphylococcus aureus* protein A, *spa*, uses a combination of the size variability of the gene due to VNTR and the variability of the sequence of the locus resulting in excellent discriminatory power (Harmsen et al., 2003; Koreen et al., 2004).

The genomes of bacterial species have different levels of diversity. Some species are considered monomorphic such as *Bacillus anthracis*, *Yersinia pestis* and the *Mycobacterium tuberculosis* complex. These bacteria are thought to have emerged relatively recently as pathogens in humans (Achtman, 2008). Typing of these species is challenging because most of the genome is highly conserved. The sequencing of housekeeping genes did therefore uncover little or no diversity and MLST was consequently not suited for these species (Achtman, 2008). Several other methods have been developed for monomorphic species, however the use of MLVA has gained general acceptance for this group of species. The analysis of CRISPR (clustered regularly interspaced short palindromic repeats) loci as a

related method has been introduced for species, e.g. *M. tuberculosis* (Pourcel and Vergnaud, 2011).

The analysis of the whole genome of strains revealed that the methods mentioned above are not able to describe the complete genetic diversity of a bacterial genome (Tettelin et al., 2005). In contrast, whole-genome sequencing allows a much deeper understanding of the evolution of a bacterial species either within the species or in comparison to other species. Genetic sequencing has become accessible for more and more laboratories since the costs have decreased dramatically. While sequencing itself has become affordable, the costs for the subsequent bioinformatic work and necessary confirmatory experiments have not changed in the same way. Therefore the assembly of a sequenced genome into contigs that allow interpretation and comparison with other genomes is still a considerable challenge. (Nagarajan et al., 2010).

1.7 Typing of GBS

Typing of GBS is done to follow the overall composition of GBS strains in a geographic region, for research purposes or for outbreak investigations e.g. in hospitals or in cattle herds. Typing of GBS starts usually with the determination of its capsular polysaccharide and, in some centers, of the strain variable Alp surface proteins. In both cases immunological methods have traditionally been used, while in later years, methods testing for the gene-content of the CPS or proteins have become available. A method combining multiplex PCR and reverse line blotting integrates typing of CPS, surface proteins and antibiotic resistance genes in one assay (Kong et al., 2005; Zeng et al., 2006). Even if these markers are combined, their discriminatory power is comparably low and therefore supplementary methods have been developed of which pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST) have been most frequently used in recent years. PFGE is used as a tool for high resolution typing to elucidate epidemiological relations between strains. Multi-locus sequence typing (MLST) was introduced for GBS in 2003 and is well suited for phylogenetic typing to follow long term developments in strain collections but might also be used for epidemiological typing (Jones et al., 2003). Both PFGE and MLST require sophisticated equipment, well trained personnel and are expensive. Newer more simple and less expensive methods have also been developed such as the MLVA described in this work or analysis of sets of single-nucleotide polymorphisms (SNPs) (Honsa et al., 2008).

1.7.1 Capsular polysaccharides

The CPS antigens can be detected by antibodies raised in rabbits or mice. The classic immunoprecipitation method was described in the 1930s (Lancefield, 1934). Fluorescent antibody tests (FAT) (Bevanger and Maeland, 1977) or agglutination of latex particles (Slotved et al., 2003) have also been used. The degree of encapsulation of GBS varies between strains; especially bovine strains can have little or no capsule (Madoff et al., 2006). Such strains tend to be difficult to type with immunological methods.

Horizontal transfer of genes that are relevant for the synthesis and assembly of the CPS may occur en-bloc and cause a switching of CPS type (Cieslewicz et al., 2005; Luan et al., 2005). This capsule switching may disrupt the phylogenetic relationship of a strain and assign it to a serotype which is not in concordance with results of other typing methods such as MLST. This has to be kept in mind when interpreting CPS typing results.

Several groups employ in-house methods for typing of CPS such as our group which uses indirect fluorescence antibody test with antibodies raised in rabbits or mice. The Statens Serum Institut in Copenhagen produces a commercially available latex based kit which is widely used by reference laboratories (Afshar et al., 2011).

The sequences of the variable CPS type-specific regions have been available the last ten years (Cieslewicz et al., 2005; Kong et al., 2002; Slotved et al., 2007). This made it possible to design PCRs for CPS genotyping both as single or multiplex PCRs (Imperi et al., 2009; Kong et al., 2005; Poyart et al., 2007). As mentioned above, PCR based typing showed that strains which were found non-typable by immunological methods, did usually harbor the genetic information for the synthesis of these polysaccharides. This problem seems to be especially common in bovine strains (Zhao et al., 2006b).

1.7.2 Surface proteins

Some groups have also used the strain variable surface proteins for subtyping of CPS types. The proteins typically appear in combination with certain CPS-types, e.g. III and R4 or Ib and Cα/Cβ. Strains with unusual CPS/protein combinations may be interesting epidemiologically. Surface protein typing can be done by immunologic methods such as immunoprecipitation or FAT. Several PCR-based methods for detection of the genes coding for these proteins have been published. Multiplexed PCRs are a convenient approach (Creti et al., 2004). As with CPS, immunologically non-typable strains usually harbor a surface protein encoding gene (Imperi et al., 2009).

1.7.3 Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) has been used for typing of GBS since the early 1990s (Fasola et al., 1993). The method is based on the macrorestriction of bacterial DNA by an endonuclease into large fragments. These fragments are then separated by gel electrophoresis where the electric field switches its direction, thereby allowing the large DNA fragments to migrate through the gel. An image of the distribution of bacterial genomic fragments in the gel is interpreted visually or by dedicated software. As for many other bacterial species PFGE is a convenient method for typing of GBS. This method has however some known drawbacks. The procedure is laborious and requires specific, expensive equipment. It is difficult to obtain comparable images of the same strain in different laboratories with PFGE. This requires rigorously standardized protocols and quality control (van Belkum et al., 2007).

For PFGE analysis of GBS the restriction enzyme SmaI, which cleaves the DNA chain relatively infrequently, is usually chosen. In an investigation of a broad selection of 78 Norwegian strains, Skjaervold et al. could distinguish 62 different types (Skjaervold et al., 2004). In an article by Tenover on interpretation of PFGE, it is suggested that at least ten fragments should be identifiable on the gel for sufficient discrimination (Tenover et al., 1995). In GBS this is in our hands often not achieved as a number of strains only produce 7-9 bands. Therefore PFGE with SmaI might be less discriminative for GBS compared to other species. Other restriction enzymes might be added for additional resolution but this would add to the complexity of the method.

1.7.4 Multi-locus sequence typing

In 2003 Jones et al. published a multi-locus sequence typing (MLST) system for GBS (Jones et al., 2003). It is based on the sequencing of 400-500 bp parts of seven housekeeping genes. Since mutations in these genes are usually not well tolerated, successful mutations occur less often than in other parts of the genome. A web-based database is available for comparison and registration of new GBS sequence types (http://pubmlst.org/sagalactiae/). Over 570 different strains are registered in the database as of January 2012. Most of these belong to clonal complexes (CC) of which four seem to be especially predominant: CC1, CC17, CC19 and CC23 (Brochet et al., 2008). One of the most important findings of MLST was the high prevalence of CC17 among strains from invasive neonatal disease. CC67 is often found in strains from bovine mastitis (Sørensen et al., 2010).

Drawbacks of MLST are its comparable high costs, a sample processing time of 3-4 workdays and the need for a considerable amount of expert hands-on work. This has led to the development of simpler methods which try to assign presumptive sequence types for the most important types by PCR or SNP analysis (Honsa et al., 2008; Lamy et al., 2006).

1.7.5 Other typing methods used

Other less frequently used methods for typing of GBS include restriction fragment length polymorphism analysis (RFLP) (Blumberg et al., 1992), ribotyping (Blumberg et al., 1992; Huet et al.), multi-locus enzyme electrophoresis (MLEE) (Quentin et al., 1995), random amplification of polymorphic DNA-analysis (RAPD) (Limansky et al., 1998; Zhang et al., 2002) and amplified *cps* restriction polymorphism analysis (Manning et al., 2005).

1.7.6 Multi-locus variable number of tandem repeats assay

Repetitive sequences which may be located both in bacterial genes or intergenic regions are common in prokaryotic genomes (Lindstedt, 2005). Repetitive sequences are challenging for the genetic copying system of bacteria and deletion or insertion of one or several repeats can occur. This leads to a different number of repeats in the bacterial descendant and consequently a different size of the locus (Figure 4). The molecular model for this phenomenon is termed slipped strand mispairing (Torres-Cruz and van der Woude, 2003). A single gene locus with such variations is called a variable number of tandem repeats locus (VNTR). If mispairing occurs it can be repaired by a DNA repair system but the efficiency of these systems is variable (Caporale, 2003). As mentioned previously repeated sequences found in intergenic regions are often polymorphic (Pourcel and Vergnaud, 2011). Repeats can also be located in a promoter region of a gene where they can influence the strength of a promoter and cause variation in gene expression (Martin et al., 2003; Willems et al., 1990). Repeat count variations inside coding regions of genes have been shown to be involved in adaption of bacteria to the host immune system as has been described for the surface proteins of GBS. If the repeat sequence is not a multiple of 3 bp, changes in the repeat number will lead to a frameshift and may result in alterations of gene functions. Other repeated sequences are well conserved which might indicate that they are vital for the correct function of a gene (Pourcel and Vergnaud, 2011). For typing purposes variations in the number of repeats results in size variations of the locus which are detectable in PCR products.

Software such as the Tandem Repeats Finder (http://tandem.bu.edu/trf/trf.html) is available for screening of genomes for repeated sequences (Benson, 1999). When more than one sequenced genome is available the sequences can be compared and polymorphism in VNTR loci uncovered in silico (Figure 4). Several VNTR loci can be combined into a multi-locus variable number of tandem repeats assay, sometimes also called multi-locus variable repeats assay (MLVA). A prerequisite for the design of a MLVA is the availability of at least one fully sequenced genome of the targeted species. It is therefore not surprising that the first MLVA published was for typing of *Haemophilus influenzae*, the first fully sequenced bacterium (van Belkum et al., 1997).

The *H. influenzae* MLVA was described in 1997, about the same time as the first MLST (Maiden et al., 1998). In the following years MLVA did not gain the same acceptance as MLST. This situation changed somewhat when MLVAs were developed for monomorphic species such as *Bacillus anthracis* and *Yersinia pestis* (Kattar et al., 2008; Keim et al., 2000; Le Fleche et al., 2001). For typing of these species MLVA was an important improvement since other typing methods were not able to uncover sufficient diversity or the results were not considered to reflect the phylogeny of the species (Achtman, 2008). MLVA has gained increasing acceptance for several enteropathogenic bacteria for which outbreak investigations are often needed and where comparisons between laboratories are of great benefit, such as *Salmonella typhimurium* or enterohemorragic *E. coli* (Lindstedt, 2011). Generally the MLVA method is reproducible, rapid, and easier to perform and produces data that are easier to analyze and share via databases than comparable methods such as PFGE or MLST. Standardization of MLVA regarding choice of loci, laboratory protocols and nomenclature is an important issue (Larsson et al., 2009; Lindstedt, 2011; Pourcel and Vergnaud, 2011). MLST in contrast is a more standardized procedure where the choice of loci is usually given and the underlying evolution of the housekeeping genes is known. This may explain the broader acceptance of MLST compared to MLVA.

Figure 4: The same VNTR locus in two fully sequenced GBS strains. In strain A909 this locus has 23 repeats, in strain NEM316 it has 19 repeats. The VNTR is used in our MLVA and designated SATR3. Underlined, primer sites. Note some sequence variation between single repeats.

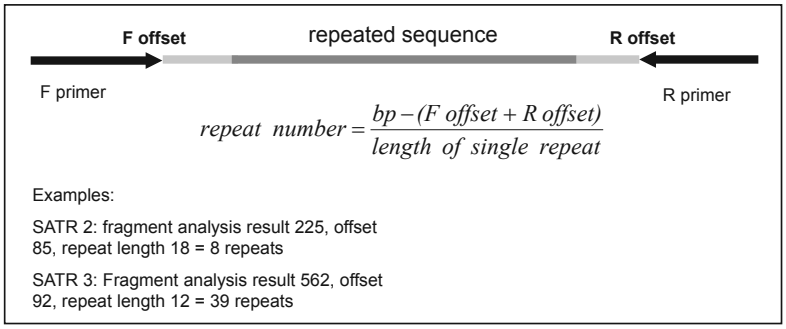
>1993465-1993889 Streptococcus agalactiae A909, complete genome
TTTCAATAGCTTTTTTAACCGCCAAGTTTCCGC
TAGTATTAACCGATTTTTTGGTTG
CTGGTTTGGCTT
CTGGTTTAGCCT
CTGGCTTAACGT
CTGGTTTAACTT
CTGGCTTAGCCT
CTGGTTTGGCCT
CTGGCTTAACGT
CTGGTTTAACTT
CTGGCTTAGCCT
CTGGTCTGGCCT
CTGGCTTAACGT
CTGGTTTAATTT
CTGGTTTAGCCT
CTGGTTTGGCTT
CTGGCTTAGCCT
CTGGTTTAACTT
CTGGCTTAACGT
CTGGCTTAACGT
CTGGCTTAACAT
CTGGTTTGGCTT
CTGGTTTAACTT
CTGGCTTAGCTT
CTGGTTTGGCTA
CTTTTTTATCTTTTAATTCTAAACTTTGTAAAGC
ATCACGTAGCTTGTTAGCTAATTGATTTGCTTG
ATCAGTGGTAATAAATTTTCCCTCT

>2089975-2090315 Streptococcus agalactiae NEM316, complete genome
AATAGCTTTTTTAGCCGCCAAGTTTCCGCTA
GTATTAACCGATTTTTTGGTTG
CTGGTTTGGCCT
CTGGTTTAGCTT
CTAGCTTAGCCT
CTGGTTTAGCTT
CTGACTTAGCCT
CTGGTTTAGCTT
CTGGCTTAGCCT
CTGGTTTAACGT
CTGGTTTAGCTT
CTGGCTTAACGT
CTGGCTTAGCTT
CTGGTTTGGCCT
CTGGCTTAGCTT
CTGGCTTAGCTT
CTGGCTTAGCTT
CTGGCTTAGCTT
CTGGTTTGGCCT
CTGGTTTGGCTT
CTGGTTTGGCTA
CTTTTTTATCTTTTAATTCTAAACTTTGTAAA
GCATCACGTAGCTTGTTAGCTAATTGAT

Technically MLVA is a fast and comparably straightforward typing method. A PCR spanning the whole repeat locus is designed by locating suitable primer sites as close as possible upstream and downstream of the repeated region. These flanking sequences should be conserved. For a MLVA, several VNTR loci may be amplified simultaneously in a multiplex PCR. Variable numbers of repeats will yield PCR products of different sizes and this can be determined either by gel or capillary electrophoresis. When using gel electrophoresis the results may be determined against reference strains with known numbers of repeats. This method will need a certain repeat size since short sequence repeats (SSR) smaller than approximately 9 bp will be difficult to distinguish on a gel and excludes multiplexing of PCRs (Pourcel and Vergnaud, 2011). Capillary electrophoresis by a genetic analyzer will generate a quite accurate estimate of the base pair count of the fluorescently labeled PCR products. Therefore also small repeats of 6 or even 3 bp can be separated in a MLVA using capillary electrophoresis. On the other hand, interpretation of PCR products larger than the upper limit of the size standard used will be difficult. The base pair count

generated by capillary electrophoresis allows an exact calculation of the repeat count. For this the offset must be subtracted from the PCR product size and the result must be divided by the repeat size. The offset consists of the primers and the sequences between the primers and the start/end of the repeat sequence (Figure 5).

Figure 5: Illustration of the structure of a VNTR and flanking sequences. The offset is the primer plus the nucleotides between primer and start/end of the repeat sequence. Examples for the calculation of a repeat number from fragment analysis for GBS-VNTR loci SATR2 and SATR3. F forward, R reverse, bp basepair count of repeated sequence.



1.7.6.1 Analysis of MLVA results

Typing methods classify the individuals of a population into groups. Both the number of groups and the size of each group generated by the method have to be taken into account to make predictions on the discriminatory power of a method. Simpson's index of diversity is a statistical tool to calculate this (Simpson, 1949). By calculating Simpson's index for different typing methods used to type the same strain collection, the methods can be compared regarding their ability to discriminate. The index should only be used when the strain collections are epidemiologically unrelated (Hunter and Gaston, 1988). The modifications of Simpson's index described by Hunter and Gaston are especially useful in small strain collections or if the diversity index is less than 0.90.

Comparison of VNTR loci or MLVA profiles may be done using different algorithms suitable for the analysis of character data sets. Most often the distance coefficient used is a categorical coefficient (Hamming's distance), e.g. given a repeat copy number of five for a locus the distance to a repeat copy number of six is considered equally distant as a repeat count of nine. The generated distance coefficient can be used for clustering for which several methods are available. In MLVA the unweighted pair group method using average linkages (UPGMA) is often used, a relatively simple algorithm assuming that the rate of evolution is

constant in all branches of the tree (Dethlefsen et al., 2011; Pourcel and Vergnaud, 2011). If a locus mutates in a plus or minus one repeat (stepwise) fashion other distance coefficients should be chosen. The mutation pattern for each VNTR locus should be determined experimentally (Vogler et al., 2007).

2 Aims of the study

The general aim of the study was to investigate the potential of molecular methods to achieve more discriminatory and robust typing of GBS. More specific aims were to:

- study the relationship between results of serotyping and genotyping of GBS surface proteins
- develop a MLVA typing method for rapid, highly discriminatory genotyping of GBS
- show the feasibility of and validate the developed MLVA method on a well characterized strain collection
- investigate the applicability of the MLVA in circumstances where epidemiologically related strains are expected.

3 Material and Methods

3.1 Strain collections

Previously well characterized collections of human GBS strains were used for papers I and II, while bovine GBS strains were investigated for paper III. In the study presented in paper I 147 strains were selected from a larger collection of strains from New Zealand and Australia. The selection included all ten CPS types and the most common CPS types were represented in higher proportions than the less common. The strains were in part (129 isolates) from invasive cases, these were also used in a previous study (Zhao et al., 2008), and in part (18 isolates) from antenatal, vaginal swabs. For paper II 126 strains were included. Of these, 113 were invasive strains from neonates born in Norway which were routinely submitted to the national GBS reference laboratory at St. Olavs University Hospital in Trondheim from other medical microbiology laboratories nationwide. Additionally nine international reference strains, two CPS type IX strains from a Danish collection and two colonizing strains from a Zimbabwean collection were included. For paper III all bovine GBS isolates (n=148) identified by the Trondheim section of the Norwegian Veterinary Institute between April 2007 and November 2010 were included. The samples were taken from individual udder quarters when considered indicated by the referring veterinarian or farmer. Additionally 39 strains from the four other sections of the Norwegian Veterinary Institute (Oslo, Sandnes, Bergen and Harstad) and from the Tine Norwegian Dairies BA, Mastitis Laboratory in Molde were included.

3.2 Typing methods

Paper I was the result of cooperation between the Centre for Infectious Diseases and Microbiology of the Westmead Institute in Sydney, Australia and the Norwegian national reference laboratory for GBS at St. Olavs University Hospital in Trondheim, Norway. The Australian group had established a gene-based typing method for GBS to detect the genes for the strain variable surface proteins of the alpha-like protein (Alp) family *bca* (encoding the Cα protein), *alp1* (Alp1), *alp2* (Alp2), *alp3* (Alp3), *alp4* (Alp 4) and *rib* (R4/Rib). Additionally the Cβ protein encoding gene *bac* was analyzed. The method was based on multiplex PCR followed by the demonstration of the PCR products by reverse line blot hybridization (Kong and Gilbert, 2006; Zhao et al., 2006a). The group in Trondheim serotyped the same strains by fluorescent antibody tests based on in-house antisera. Of the seven antisera used, sera reacting with Cα/Alp1, R3, R4/Alp3 common and Cβ were monoclonal antibodies raised in mice (Bevanger et al., 1992; Kvam et al., 1999; Maeland et al., 2004; Naess et al., 1991) and sera reacting with Alp2/3 common, Alp2 specific and R4 specific were polyclonal antibodies raised in rabbits (Maeland et al., 2004; Moyo et al., 2001).

Paper II described the construction and assessment of a MLVA for GBS. All strains used in the study were typed for CPS, surface proteins and MLST. For the Norwegian strains the results for these three markers were known from a previous study (Bergseng et al., 2009). The MLVA was developed by exploring the three fully sequenced and annotated genomes of GBS reference strains A909, NEM316 and 2603V/R for repeated sequences using the Tandem Repeats Finder program and other software. Eighteen candidate loci were identified and tested by PCR and gel electrophoresis or using the Agilent 2100 Bioanalyzer with the DNA 1000 chip (Agilent Technologies, Santa Clara, CA). This led to the selection of five VNTR loci which were combined to a MLVA. In a further step a multiplex-PCR including all five loci was constructed using the Qiagen multiplex-PCR kit (Qiagen, Hilden, Germany). The forward primers for the five loci were fluorescently labeled and the nucleotide count of the PCR product was estimated in a capillary electrophoresis instrument (ABI 3130xl Genetic Analyzer; Applied Biosystems, Foster City, CA) using the LIZ1200 size standard. This standard allows estimates of PCR product sizes up to 1200 bp. In paper III, the same MLVA methods as in paper II was used except for a modification of the multiplex-PCR. Here the SATR5 locus was amplified in a separate PCR reaction. In this paper a repeat count of zero indicated that no PCR product was detected(Figure 1, Paper III).

3.3 Analysis of results

In paper II Simpson's index of diversity was calculated. Phylogenetic trees were generated with the Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium) version 6.0 (paper II) and 6.1 (paper III). Phylogenetic trees were generated using the Bionumerics software. The algorithm used was the unweighted pair group method with arithmetic means (UPGMA) and categorical values.

4 Results

4.1 Paper I

Classic serotyping and genotyping of the strain variable GBS surface proteins were compared in a selected panel of 147 human strains. The comparison of the two methods employed is complex and reflects the complicated immunology of these proteins. At least one surface protein was detected by serotyping in 123 (83.7%) of the strains, while a surface protein encoding gene was detected in all but one of the strains (146 strains, 99.3%). In total, 122 of 147 strains were typable by both antibody-based and molecular methods. Results were concordant for 90 (73.8%) strains, partially concordant for 29 strains (23.8 %) and discordant for three (2.5 %). When the serotyping result was Cα/Alp1, either *bca* or *alp1* was present. The *bca* gene was detected in 41 isolates and *alp1* in 30. Of these 71 strains, 55 (77.5%) were serotyped as Cα/Alp1. The monoclonal antibody used cannot distinguish between Cα and Alp1. Cβ was identified in only nine of 31 isolates in which the corresponding gene, *bac* was detected. The discordant results occurred mainly with the polyclonal antibodies detecting Alp2 and Alp2/3 common (Table 1, Paper I). Ten of 24 non-serotypable strains contained *rib* which means that the polyclonal antibodies did not recognize the protein in 22 of 32 strains genotyped as *rib*. We detected R3, for which no corresponding gene has been identified, in five isolates (3.4%).

The genotyping approach did very well in identifying the genes for surface proteins. The problem of cross-reactivity which hampers the performance of serotyping is not an issue in genotyping. Especially typing problems in both Alp3, which is an immunologic chimera of Alp2 and R4 without known antigenic sites of its own, and of Cα and Alp1, which are hardly distinguishable immunologically (Brady et al., 1988; Kvam et al., 2011), were not encountered. The only non-typable strain by genotyping posed also difficulties in the serotyping approach and no definite type could be assigned (result: Alp2 or 3).

4.2 Paper II

4.2.1 Construction of a GBS-MLVA

In this study a MLVA was constructed consisting of five VNTR loci in the genome of GBS. The applicability of the MLVA was demonstrated on a collection of 126 well characterized invasive strains. The construction of the MLVA involved several steps. An initial in silico analysis of the three complete genomes of strains A909, NEM316, and 2603V/R identified 18 candidate loci which were analyzed further by designing PCR primers and examining these in eleven selected strains. Seven loci were not found suitable for further analysis and were therefore excluded. In six of these no or little diversity was found while problems to obtain a PCR product led to the exclusion of the last. The remaining eleven loci were tested in all strains. In this part of the study seven loci were found to have three or more alleles. Of these the five most diverse were chosen. In each of the three loci designated SATR1, TR9 and TR13 three different alleles were found among the 126 strains. Based on the results of the calculation of Simpson's index of diversity for each locus only SATR1 was included in the MLVA (Table 2, Paper II). For each of the four other loci included in the MLVA four or more alleles were detected.

The location of the five loci in the genome of the three reference strains 2603V/R, NEM316 and A909 and the designation of the loci are given in Table 1. The five loci had different characteristics which can be summarized briefly as follows:

1. SATR1 is a CRISPR-like region with 16 bp direct repeats and 44 bp spacer regions, each repeat/spacer was therefore 60 bp long. We did only find three different alleles in our collection of human strains and a fourth in our bovine collection, while a Russian group encountered eight different types in their work with the same region (Rozhdestvenskaya, 2008).
2. The repeats in the SATR2 locus had a repeat size of 18 bp. Nine different alleles were found. Strains devoid of a PCR product were also considered as one type; this result was encountered in 14 of the 126 strains.
3. The SATR3 locus had a repeat unit of 12 bp. In this strain collection 19 different alleles were found, rendering SATR3 the second most diverse locus. The repeated sequence is part of a predicted surface-anchored protein designated BibA (Santi et al., 2007). Large PCR products, between 736 and 750 bp with the chosen primer set, were encountered in 31 strains. Sequencing of PCR products demonstrated that the strains with the largest products had had two copies of an insert of 216 bp. All but one of

these strains were of MLST clonal complex 17. This insert has also been proposed as a target for a PCR for the rapid presumptive identification of ST17 (Lamy et al., 2006). In 25 strains no PCR product was found, most of these were of serotype III/R4 and of clonal complex 19.

4. Amplification of the SATR4 locus resulted in the smallest PCR products of the five loci. The underlying repeat of 18 bp was quite degenerated; in some strains part or all of the repeats consisted only of 15 bp. For convenience of typing all alleles were assigned as times of 18 bp in the calculation of repeat numbers. A PCR product was observed in all strains and five different types were present in this collection. The repeat is located in the *pcs*B gene, encoding a protein found to be important for cell wall separation (Reinscheid et al., 2001).
5. The SATR5 locus was the most diverse separating the strain collection into 24 different types. The underlying repeat is rather large with 48 bp and strains with more than 20 repeats were found in some strains. Five strains had more than 21 repeats, resulting in PCR products which exceeded the size standard limit of 1200 bp. In the analysis they were considered as one type with the arbitrary repeat number of 50 although they showed diversity among each other by standard gel electrophoresis. When the PCR product was equivalent with the offset (21 strains) the strain was usually of MLST19. A result of six repeats was found in 22 strains, 18 of which belonged to ST17. SATR5 is located in the gene encoding the fibrinogen binding protein FbsA (Rosenau et al., 2007; Schubert et al., 2002).

Table 1: Location of the five MLVA Loci. Position and annotation in reference genomes.

	2603V/R	NEM316	A909	Note
SATR1	142905-143227 Hypothetical protein SAG0134	142937-143139 Intergenic region	152958-153160 Hypothetical protein SAK_0192	CRISPR neighbouring transposase (sequence inversed)
SATR2	666413-666676 Hypothetical protein SAG0677	Not found	745915-746218 Hypothetical protein SAK_0805	
SATR3	2043303-2043801 Pathogenicity protein SAG2063	2090028-2090347 Surface adhesion protein gbs2018	1993422-1993889 Intergenic region	Upstream of a neighbouring translocase (sequence inversed), designated BibA
SATR4	29490-29744 PcsB protein	29563-29817 PcsB protein	29486-29698 PcsB protein	Involved in cell wall seperation
SATR5	1062519-1062709 Intergenic region (no repeats)	1132283-1133330 Hypothetical protein gbs1087	1118169-1119009 Fibrinogen binding protein SAK_1142	Sequence inversed, FbsA protein

4.2.2 Comparison between MLVA, serotyping, and MLST

MLVA analysis using the five proposed loci differentiated the 126 strains into 70 different types, considerably more than MLST which differentiated them into 36 STs. The two largest groups of strains with identical MLVA profiles consisted of 19 and 13 strains, respectively. Of the former, 16 belonged to ST17 and 9 of the latter to ST19. All 27 of the ST17 strains in this work clustered together but were resolved into 12 different MLVA types within this cluster. Apart from the ST17 MLVA profile present in 16 strains, the eleven remaining profiles were found in single strains only (Fig. 2, Paper II). Similarly, all 11 CPS III/ST19 strains were assigned to the same MLVA cluster and resolved into four MLVA types; 7 of the strains had identical profiles. Most of the type V strains belonged to ST1 (21 out of 30 type V

strains); 20 of these 21 were in a cluster consisting of 12 MLVA types, mostly because of heterogeneity in SATR5.

4.3 Paper III

In this study 187 bovine GBS strains were investigated by genotyping of CPS and surface proteins and by MLVA. The following CPS types were found: Ia (seven strains, 3.7 %), Ib (six strains, 3.2 %), II (three strains, 1.6 %), III (three strains, 1.6 %), IV (64 strains, 34.2 %), V (101 strains, 54.0 %), IX (one strain, 0.5 %) and two strains (1.1 %) were non-typable. CPS type V and type IV accounted together for 88 % of the 187 strains, an overrepresentation compared to other strain collections. Genes for five of the six strain variable alpha-like proteins were found except the rarely encountered Alp4; additionally the gene for Cβ was detected. The combination of CPS and protein typing separated the strain collection into twelve different types (Table 2, Paper III).

The MLVA was equally applicable on bovine strains as on human strains. The 187 strains were differentiated into 37 MLVA profiles (Figure 1, Paper III). In 29 of 34 farms all GBS isolates on each farm had identical MLVA profiles, different from strains from other farms. On the remaining five farms more than one profile was encountered (Table 3, Paper III). Usually one profile predominated while a single strain with a divergent repeat number in one locus was observed. One MLVA profile was found on three farms in the same municipality. This was presumably the result of transmission due to cooperation between these farms. Three other profiles were found on several farms, however these were located in different counties and an epidemiological connection was regarded unlikely.

Automated milking systems (AMS) were used on 12 of the 34 farms (35%). Not unexpectedly, those farms which had invested in AMS had bigger herd sizes (61.4 versus 27.7 cows). Of the 187 strains, 133 were provided by farms with AMS (71%). Also the AMS farms provided 0.18 isolates per cow against 0.09 isolates per cow in non-AMS farms. On average, 11 strains were available from farms with AMS compared to 2.45 strains from those with conventional milking.

One farm was represented with 48 samples collected over a period of almost three years. Four different MLVA profiles were found among these with polymorphisms occurring in SATR5 of 13, 15, 17 and 50 repeats, respectively. In the first part of the observation period isolates with 17 repeats occurred in 19 samples (sampled until September 2009), replaced

later by isolates containing 13 repeats (in 27 samples, collected from August 2009 onwards). The other two profiles were only found in single strains.

Seven of the MLVA profiles from cattle were also encountered in our recent study on human strains (Radtke et al., 2010). In a sub-analysis the relatedness of bovine and human strains was investigated. 47 of the bovine strains (representing at least one strain per farm and MLVA profile) and seven human strains were examined for lactose fermentation, bacitracin sensitivity, and presence of the *scp*B gene. In this analysis all but one of the bovine strains fermented lactose, while none of the seven human strains did. The *scpB* gene was found in about two-thirds of our bovine strains while other investigators found that a negative result was strongly associated to bovine strains (Franken et al., 2001; Sørensen et al., 2010). Also Bacitracin was less reliable in differentiating between bovine and human strains.

5 General discussion

In this work we investigated selected typing techniques for GBS, i.e. serotyping and genotyping of strain variable surface proteins and the MLVA developed in this study. The typing of bacteria within a species is usually done for surveillance, epidemiologic or research purposes. Which methods are chosen will depend on the scientific problem and the methods available. Different methods have different resolution; i.e. the ability to classify a strain collection into groups. A basic typing method, e.g. the typing of the GBS polysaccharide capsule, will separate GBS strains into only ten different groups, while the perhaps ultimate typing technique of the future, whole genome sequencing, must be expected to characterize almost no strains as identical. This will lead to an extreme version of the problem common for high resolution methods such as MLVA or PFGE, i.e. how much divergence between results can be accepted before strains are no longer considered related or closely related, a question discussed by van Belkum (van Belkum et al., 2007) or for the case of PFGE by Tenover (Tenover et al., 1995). If strains are obtained over a longer period of time, such as the strains in our paper III which were sampled over three years, a greater divergence of strains should be tolerated in the comparison of strains since some degree of mutation has to be expected over time.

Technical advances continuously generate new microbiological typing methods which have to be compared with established techniques in order to investigate their congruence and to secure continuity in the typing of bacteria. Such comparisons will enable the scientific

community to discuss allocations of strains into concordant groups on the background of different typing methods.

In this study we compared immunological serotyping of surface proteins with newer genotyping (paper I). Further we aimed at and succeeded in developing a MLVA method for GBS and compared its typing results with genotyping of capsular polysaccharides, surface proteins and MLST (paper II) and applied it in an investigation of strains from GBS mastitis in cattle in Norway (paper III).

5.1 Typing of surface proteins

The main objective of paper I was to investigate if immunological serotyping and genotyping of GBS surface proteins produced concordant results. Ideally both methods should show complete concordance. This however was not to be expected since non-typable strains are regularly encountered in serotyping and immunological cross-reactivity is a well known problem in typing of Alp proteins (Lindahl et al., 2005). A previous study addressed this problem for a limited number of surface proteins (Persson et al., 2008).

The Department of Medical Microbiology at St. Olavs University Hospital in Trondheim holds an extensive collection of antibodies against the strain variable surface proteins of GBS. For the current work, this was paired with a thoroughly designed genotyping approach for these surface markers by the contributors from Australia (Zhao et al., 2006a). Genotyping is usually a straightforward test and identifies the gene for the protein in question if the corresponding PCR is carefully designed. Apart from the Alps the study also targeted two proteins which are not part of the alpha-like protein family: Cβ and R3. While the former is well known and usually part of typing of surface proteins, R3 is less often looked for and in contrast to the others the genetic sequence is not known and therefore no PCR is available.

For typing of GBS surface proteins the study indicates that genotyping should be preferred, as it leaves only few strains non-typable and gives usually unambiguous results. Serotyping may be used as a supplementary method. Immunologically the phenomenon of non-typability or cross-reactivity might reflect problems with the specificity of the antibodies or other assay related problems. However, this could also reflect changes in the proteins such as deletion of repeats or mutations at antigenic sites leading to immunologic variation.

5.2 MLVA typing of GBS

The method of multi-locus variable number of tandem repeats analysis has several advantages compared to other typing methods. MLVA uses PCR assisted amplification of repeat loci and size assessment of the PCR products by electrophoresis. These are fast and comparably straightforward methods using less complicated laboratory protocols compared to e.g. MLST or PFGE. The equipment needed for MLVA is available in many microbiological laboratories, especially when gel electrophoresis is used. The results can be given in repeat count, particularly if PCR product size is analyzed by fragment analysis. Fragment analysis by capillary electrophoresis gives an estimate of the PCR product size in base pairs. This can be computed into the repeat count which is easily exchangeable between laboratories (Figure 5). Websites for exchange of these data have been established for several bacterial species. The resolution achieved by the method is depending on the variability and number of VNTRs used. A published MLVA has to be considered as a selection of possible loci and further investigation of the genome of a bacterial species might find additional loci which can be combined with previously described into an even more discriminating assay.

These characteristics render MLVA a tool which is especially well suited for investigation of outbreaks. For phylogenetic typing or other issues, where less discriminative assays might be preferred, MLVA might also have a place, particularly when databases are accumulating many strains/types and consensus on what to consider as related strains has been reached (Pourcel and Vergnaud, 2011). Although variable genetic areas are often associated with repeats, the speed of mutation can be very different. Depending on the intention of the analysis, selecting VNTRs for phylogenetic studies or the evaluation of long-term epidemiology might be possible. A selection of slowly mutating loci can result in a MLVA with characteristics closer to MLST. Even without such a selection the phylogenetic trees in paper II showed that the strains are grouped with good congruence with the results of the phylogenetic reference method MLST, in which clonal complexes are usually thought to reflect phylogenetic linages. The proposed MLVA could theoretically be used to assign presumptive clonal complexes of MLST.

In Paper II we investigated the stability of loci by passaging three strains 40 times in vitro without observing variability. Findings made in paper III indicated that the stability of VNTR loci in GBS and their mutation rate could be observed in cattle in vivo. In this study of Norwegian bovine GBS we had the opportunity to investigate strains which were collected on distinct farms over almost three years. We detected repeat count changes on five farms. On

four of these, changes appeared at only one locus, on the fifth farm with two available samples, differences at two loci were detected. The most notable example for variability was observed in the SATR5 locus at a farm represented in the study by 48 samples. We propose that the term Single Locus Variants (SLV), by analogy to MLST, may be applicable for such MLVA results.

The epidemiology of GBS in humans has to take into account the large proportion of gastrointestinal carriers of the bacterium. Disease arises from the translocation of the bacterium into usually sterile compartments such as blood, cerebrospinal fluid or synovial fluid. The bacterium is ubiquitous among humans and outbreaks are not common. Small outbreaks have occurred on maternity wards or in dialysis units; however they are usually limited to a small number of cases (MacFarquhar et al., 2010). In animals on the other hand outbreaks of GBS disease are common, especially as mastitis in cattle (Keefe, 1997; Zadoks et al., 2011). The collection of strains used in paper III reflects an increased occurrence of GBS as a cause of mastitis in Norwegian cattle in recent years. The MLVA performed excellently in discriminating the 187 bovine strains into 37 different profiles. Almost every farm had its own profile and a farm-specific profile was usually not encountered on other farms, as mentioned above and in the paper. This is in accordance with the information that animals are usually isolated at their farm without the possibility of direct contamination from other farms. Farms with long lasting GBS problems might therefore be used to study the microevolution of a GBS strain genetically. For the case of MLVA, this might give information about the in vivo stability of the selected VNTR loci under immunological pressure.

A French group has recently proposed a MLVA for GBS consisting of six VNTR loci (Haguenoer et al., 2011). Interestingly three of their loci are also included in our MLVA. An additional VNTR selected by them was investigated by us (TR9 in paper II), but not included into our MLVA. Haguenoer et al. also compared their MLVA with MLST results. While our paper II focuses on pediatric invasive strains, their selection of 186 strains was broader with human invasive, human colonizing and bovine strains. Their MLVA was as well able to group strains with good congruence to MLST results. The group emphasizes that they looked for VNTRs which were present in all their strains. This was done to avoid problems “in terms of resolution and reproducibility”. Their argument for this was that a negative result could occur either if the repeat locus is lacking or the primers fail to anneal due to variable flanking regions. In our opinion this only represents a problem if different primers are used by different groups. A negative result is also a valid typing result of a VNTR locus. When using

different primer pairs for the same VNTR it should be ascertained that they achieve identical results. An eventual cumulative MLVA from these two works should have consensus primer sets for those loci which are present by both approaches.

5.3 GBS and bovine mastitis

The epidemiology of GBS infections in bovines is strikingly different from epidemiology in humans. The route of GBS transmission into herds is not obvious. Colonization of the gastrointestinal tract does not seem to occur and the udder is thought to be the only location of GBS infection (Agger et al., 1994; Zadoks et al., 2011). Therefore eradication of GBS from bovine herds might be possible and has been widely achieved through infection control measures in the 1970s and 1980s in Scandinavia (Katholm, 2010b; Zadoks et al., 2011). The question of evolution of human and bovine GBS is of importance in this setting and for human disease. If bovines are a reservoir for human disease this should be addressed in the discussion of prevention of human disease. If human strains infect bovines, total eradication of bovine disease will be difficult to achieve. Several investigators addressing the relationship of human and bovine GBS tend to conclude that they are separate entities (see chapter 1.4.3). Experiments from the 1980s with artificial infections of bovine udders suggest that human strains could cause symptomatic mastitis but might not lead to subclinical infection (Jensen, 1982).

A reemergence of GBS mastitis in Scandinavian farming has been observed over the last 10 years (Katholm, 2010a; Persson and Landin, 2009; Zadoks et al., 2011). This is best documented in Denmark, but has also been observed in Norway and Sweden. Several possible reasons for this have been mentioned. Herd size has increased and GBS eradication is more complex on such farms. Another development involves free stalls instead of boxed stalls, facilitating more physical contact between the animals. Finally automated milking systems have been introduced and are suspected to be associated with the reemergence of GBS mastitis (Persson and Landin, 2009).

5.4 Limitations of the study

In paper II we chose to compare our MLVA with MLST. Considering that MLVA is foremost a tool for high resolution typing which is well suited for outbreak investigation, a comparison of the method with PFGE might have been equally interesting. Usually PFGE

will result in a higher resolution compared to MLST and might therefore be better suited for a comparison of epidemiological typing methods. As mentioned above, PFGE in GBS with the restriction enzyme Sma1 has in our hands not given the resolution expected from this method. The MLST method used here is well suited for phylogenetic comparisons and the most common used method for this kind of questions in later years (Feil, 2004; Pourcel and Vergnaud, 2011). MLVA and MLST clustered the strains into similar groups and MLVA could be used to assign a presumptive clonal complex to a strain, given more experience with MLVA.

The problem of mutation frequency and pattern of VNTRs was addressed both in Paper II and III but a thorough study of these questions should be performed with dedicated in vitro experiments. The investigation of farms with long lasting GBS problems might be an alternative way to observe this in vivo.

In paper III the strains were submitted to the microbiological laboratory at the farmer's or veterinarian's choice. It is therefore not known if the strain collection was representative and definite conclusions about the extent of the problem in Norway could not be made. However, GBS mastitis was only very rarely encountered in the last two decades and the frequency of GBS isolations in the Trondheim laboratory of the Norwegian Veterinary Institute was considerably higher than in the other regions. Further prospective studies should be done. In particular such studies might compare farms with and without AMS.

6 Conclusions

- Typing of GBS surface proteins by immunological and molecular methods provides concordant or partially concordant results in the large majority of strains.
- Genotyping is superior to serotyping since it is able to type almost all strains and leads to less ambiguity.
- A MLVA typing scheme for GBS was designed based on five VNTR loci.
- The MLVA performed excellently with very good discrimination. MLVA typed the strains into epidemiological groups comparable to MLST and typing of CPS and surface proteins.
- MLVA analysis of bovine GBS allocated a specific genotype to almost every farm while isolates from one farm were always identical or closely related.

7 Future aspects

Routine typing of GBS surface proteins should be performed by PCR. However, for confirmation of ambiguous PCR results and for immunological studies serotyping should be available as a supplementary method. The gene encoding the R3 protein should be identified.

The MLVA method presented in this work should be validated further and used for typing of strain collections from other geographic regions. Although some sequencing of PCR products from the five MLVA loci was done during the development of the method, sequencing of further alleles is desirable for verification and further study of the diversity of the loci. The method presented here should be compared with the method published by Hagenouer et al. Ideally consensus primers for the three common loci should be agreed upon. Further a common database on the internet with a consensus designations should be established. The mutation pattern for each locus should be determined since this would have implications for phylogenetic analysis.

8 Referanser

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Note

Identification of surface proteins of group B streptococci: Serotyping versus genotyping

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ABSTRACT

We compared serotyping to genotyping of group B streptococcal (GBS) surface proteins in 147 Australasian isolates. Results were concordant for the two methods in 73.8% of 122 isolates, discordant for three and partially discordant for 29 isolates. For the purpose of epidemiological typing of GBS, genotyping is superior to serotyping.

Streptococcus agalactiae (group B streptococcus, GBS) is an important human pathogen, particularly perinatally. Capsular polysaccharides (CPS) and surface proteins are important antigens, which are used extensively for basic epidemiological serotyping (Cole, 2008; Johri et al., 2006; Nizet and Rubens, 2006; Pannaraj et al., 2008) and serosubtyping (Kong et al., 2002; Kvam et al., 1995), respectively. *S. agalactiae* strains almost always express one of a family of surface-anchored, so-called alpha-like proteins (Alp), of which Cα, Alp1, Alp2, Alp3 and R4/Rib have been studied extensively. They are characterized by long repetitive elements and mosaicism due to recombination, resulting in sharing of epitopes and antigenic cross-reactivity (Lachenauer et al., 2000). This cross-reactivity suggests that a common protein vaccine could provide broad spectrum protection against GBS disease (Seifert et al., 2006). Cß is an IgA binding surface protein, which does not belong to the Alp family, but is also used for typing, and a potential vaccine component (Yang et al., 2007).

Serotyping methods are laborious and some isolates are non-typable, despite harbouring surface protein genes. However, serotyping does not require knowledge of the genetic sequences encoding the proteins or their flanking regions. For example, this is unknown for R3, although it is identifiable with antisera. Methods based on demonstration of genes encoding surface proteins have been developed and are becoming standard in GBS-protein typing, but there has been little direct comparison of methods (Kong et al., 2002). The aim of this study was to compare serotyping with antisera against most known GBS surface proteins with PCR-based genotyping, using a representative panel of GBS isolates.

A panel of 147 was selected from a large collection of well-characterized GBS isolates to include all 10 CPS serotypes (Ia, Ib, II–IX), expressing the main protein antigens Cα, Cß, Alp1, Alp2, Alp3 and R4/Rib (Kong et al., 2008; Zhao et al., 2008). The majority (129 isolates) had been isolated from blood or cerebrospinal fluid (Zhao et al., 2008) and the remainder from vaginal swabs collected for routine antenatal screening by laboratories in Australia or New Zealand. Isolates were stored in skim milk broth at −80 °C and subcultured on to blood agar before testing. Genotyping was performed in Sydney, Australia and isolates were sent on chocolate agar slopes to Trondheim, Norway, for serotyping; both methods were performed without knowledge of the results of the other.

Serotyping was performed by whole-cell, indirect immunofluorescence assay with in-house antibodies as described elsewhere (Bevanger and Maeland, 1977). Murine monoclonal antibodies (mAb) against Cα/Alp1(Bevanger et al., 1992); Cß (Naess et al., 1991); R3 (Kvam et al., 1999) and R4/Alp3 common (Maeland et al., 2004) and rabbit polyclonal antibodies to Alp2/3 common (Moyo et al., 2001), R4 specific and Alp2 specific (Maeland et al., 2004) were used. Fluorescent anti-murine or anti-rabbit immunoglobulin conjugates were used as recommended by the manufacturer (Dako A/S, Glostrup, Denmark). Fluorescence was assessed on a Nikon Eclipse fluorescence microscope and graded from 0 to 3+, where scores of 2+ and 3+ were interpreted as positive. If isolates reacted with Alp2/3 common antiserum, they were further tested to distinguish Alp3 from Alp2 using polyclonal Alp2

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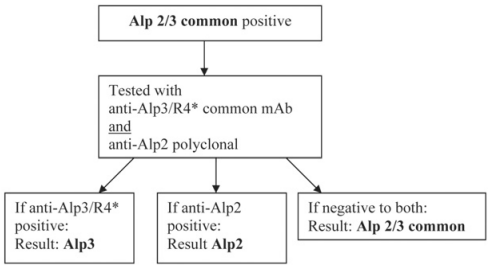

Fig. 1. Algorithm for testing Alp 2/3 common positive isolates.

and mAb R4/Alp3 common antibodies (Fig. 1). The Cα/Alp1 mAb, originally considered specific for Cα, is now known to recognize both Cα and Alp1 (unpublished data).

A multiplex PCR-based reverse line blot hybridization assay (mPCR/RLB) was used for identification of protein and CPS genes, as previously described (Kong and Gilbert, 2006; Zhao et al., 2006). Briefly it involves, after DNA extraction (Wang et al., 2008), the use of primer pairs targeting GBS (species-specific control) and seven protein encoding genes: *bca* (Cα), *rib* (R4/Rib), *alp1*/ε (Alp1/epsilon), *alp2* (Alp2), *alp3* (Alp3) and *bac* (Cβ). Amplicons are detected by hybridization with target-specific probes fixed to a nylon membrane and resolved by chemiluminiscence. In the same assay the CPS type was determined as described previously (Kong et al., 2005). Serotype IX was identified separately (Kong et al., 2008; Slotved et al., 2007).

Serotyping detected at least one protein in 123 of 147 isolates (84%) and 24 (16%) were non-typable (Table 1). The most common were Cα/Alp1, in 55 isolates (37%) and Alp2, Alp3 or Alp2/3 common in 46 (31.2%). R4/rib was found in 22 (15%) isolates, R3 in five (3.4%). Cß was detected in nine (6.1%) isolates, seven of which expressed at least one additional protein, most commonly Cα/Alp1. Overall, eleven isolates (7.4%) contained more than one protein (Table 1). Genotyping detected at least one protein gene by mPCR/RLB in all but one of 147 isolates tested (Table 1). The only combination detected was *bca* and *bac*. Typing of CPS was also done by mPCR/RLB and the surface proteins detected generally corresponded with the CPS types with which they are usually associated (Table 2).

Table 1
Results of serotyping and genotyping of 147 group B streptococcal isolates.

	Genotyping							
Serotyping	alp1	alp2	alp3	bca	bca+bac	rib	neg	Total
Alp2		2	1					3
Alp3			17					17
Alp3, R3			2					2
Alp2/3 common	2	1	16					19
Alp2/3 common, R3							1	1
Cα/Alp1	23			6	19			48
Cα/Alp1, Alp2					1			1
Cα/Alp1, Cß					3			3
Cα/Alp1, Cß, Alp2					2			2
Cα/Alp1, Cß, R3					1			1
Cß					2			2
Cß, Alp2/3 common					1			1
R3			1					1
R4/Rib						22		22
Negative	5	1	2	4	2	10		24
Total	30	4	39	10	31	32	1	147

Grey fields: concordant results in both typing methods.

Table 2
Distribution of capsular polysaccharide serotypes and their associated surface proteins.

Capsular serotype	Total	Protein encoding gene (*n*=)
Ia	28	*alp1* (23), *alp2* (2), *bca* (2), *bca*+*bac* (1)
Ib	30	*bca*+*bac* (29), *bca* (1)
II	13	*rib* (7), *alp3* (5), *bca* (1)
III	28	*rib* (25), *alp2* (2), *alp3* (1)
IV	5	*alp1* (4), *alp3* (1)
V	32	*alp3* (30), *alp1* (1) , negative (1)
VI	7	*bca* (5), *alp1* (2)
VII	2	*alp3* (2)
VIII	1	*alp3* (1)
IX	1	*bca*+*bac* (1)
Total	147	

122 of 147 isolates were typable by both antibody-based and molecular methods. Results were concordant for 90 (73.8%) isolates, discordant for three and partially discordant for 29 isolates (Table 1). When Cß, Alp2, Alp3 or R4/Rib were found by serotyping, genotyping always detected the corresponding gene. When the serotyping result was Cα/Alp1 either *bca* or *alp1* was present; *bca* was detected in 41 isolates and *alp1* in 30 and, of these, 55 were serotyped as Cα/Alp1 (77.5%). The antibody used cannot distinguish Cα and Alp1 but whether they are distinct immunologically remains to be demonstrated. Cß was identified in only nine of 31 isolates in which the *bca*/*bac* combination was detected. Discordant results occurred mainly with Alp2 and Alp2/3 common polyclonal antibodies (Table 1). Ten of 24 non-serotypable isolates contained *rib*. We detected R3 – for which no corresponding gene has been identified – in five isolates (3.4%), one of which was the only one that was nontypable by mPCR/RLB; the other four expressed other proteins, in addition to R3 and three of these contained the corresponding genes.

GBS surface proteins are composed of modular subunits (Lachenauer et al., 2000) in different combinations, which interfere with the specificity of some antibodies. Genotyping can resolve this cross-reactivity, which is highly desirable in epidemiological typing. In a recent study of GBS protein typing, Persson et al. studied 297 Swedish isolates (Persson et al., 2008), using mAbs against Cα, Cß and R4/Rib and a multiplex PCR to detect *bca*, *alp1*/ε, *rib* and *alp2*/*3*. Serotyping and genotyping were performed by the same laboratory that did the serotyping in this study. The R4/Rib mAb cross-reacted with several strains containing *alp3*, as predicted from previous studies (Maeland et al., 2005).

In the present study, eight antisera were used, including an approach to resolve cross-reactions with Alp2/3 common antisera (Fig. 1). The absence of cross-reactions between Alp2/3 and R4/Rib confirmed the specificity of the polyclonal R4 antibody. Most isolates with *alp3* reacted with either Alp2/3 common or Alp3, one with Alp2 and three with none of the antisera. To our knowledge, Alp3 is a chimera of Alp2 and R4/Rib and has no unique binding site, but binds only antibodies which also react with other GBS surface proteins (Maeland et al., 2005).

The polyclonal R4/Rib antibody failed to recognize a high propotion of *rib*-positive isolates, many of which were non-serotypable. Whether this represents low-level protein expression or reduced antibody binding caused by antigenic variation was not investigated. The latter has been demonstrated for Cα, in which variable repeat numbers cause antigenic variation (Gravekamp et al., 1996).

R3 is one of the classical R proteins (Wilkinson, 1972) but has not been sequenced. Therefore genotyping is unavailable. The frequency of R3 (3.4%), among these Australasian isolates is comparable to that (6.5%) among Norwegian isolates (Kvam et al., 1999), but lower than that (>20%) in Zimbabwe (Mavenyengwa et al., 2008; Moyo et al., 2002).

Mosaicism and structural variation of GBS surface proteins are important features for immunological and vaccine research, but cause

discriminatory problems for epidemiological typing. We have shown that GBS surface protein genotyping is superior, as almost all isolates are typable and the results are unambiguous.

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Paper II

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Rapid Multiple-Locus Variant-Repeat Assay (MLVA) for Genotyping of *Streptococcus agalactiae*[▽]

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Several methods have been used for typing of *Streptococcus agalactiae* (group B streptococci [GBS]). Methods currently in use may provide inadequate resolution (e.g., typing of capsular polysaccharides and surface protein) or are labor-intensive and expensive (e.g., multilocus sequence typing [MLST] or pulsed-field gel electrophoresis). This work describes the construction and use of a multiple-locus variant-repeat assay (MLVA) on 126 well-characterized human GBS strains, consisting mostly of invasive Norwegian strains and international reference strains. Based on *in silico* whole-genomic analysis of the genomes of strains A909, NEM316, and 2603V/R, 18 candidate loci were selected and investigated by PCR. Eleven loci showed diversity, and the five most diverse loci were used for the construction of an MLVA, consisting of a multiplex PCR followed by fragment analysis with capillary electrophoresis. The assay generated clusters which corresponded well with those observed by other methods. However, it provided a considerably higher degree of diversity, with 70 different MLVA types compared to 36 types generated by MLST. Simpson's index of diversity for the 5-locus MLVA was 0.963, compared to 0.899 for the MLST in this strain collection. MLVA results will generally be available within 2 days, which is usually faster than MLST. In our hands, MLVA of GBS represents a rapid, easy, and comparably inexpensive method for high-resolution genotyping of GBS.

Streptococcus agalactiae is the only species within the group B streptococci (GBS) of Lancefield's classification of hemolytic streptococci. It is well recognized as a human pathogen, especially for causing septicemia, meningitis, and other serious invasive disease in neonates; it is also associated with stillbirth. In addition, it is known to cause serious maternal infections and serious infections in elderly and immunocompromised patients.

Different approaches for typing of GBS have been developed through the years. Most commonly, GBS strains are assigned to one of the 10 capsular polysaccharide (CPS) types. Typing of surface proteins and analysis of the antibiotic resistance pattern can add further resolution to typing of phenotypic features. Examination of CPS or surface proteins can be done by immunological methods or, more recently, by detecting the corresponding genes by PCR (6, 8, 10). Multilocus sequence typing (MLST) was developed to study the long-term evolutionary development of a species. The total number of GBS sequence types (STs) is currently around 490 (http://pubmlst.org/sagalactiae/). Pulsed-field gel electrophoresis (PFGE) based on macrorestriction fragment analysis of genomic DNA is considered highly discriminatory. For GBS, the restriction enzyme SmaI is often used, generating only 8 to 12 restriction fragments, which may be suboptimal with respect to discriminating capacity (2, 19, 21). Since PFGE is an image-based method, it may be difficult to compare results between different laboratories. Both MLST and PFGE are labor-intensive techniques requiring experienced personnel and usually several days of work before results are available.

DNA loci consisting of repeated sequences are widespread throughout the genome of bacteria and have a tendency to vary in repeat number from strain to strain, so-called "variable number of tandem repeats" (VNTR). The combination of several VNTR loci in an assay (multiple-locus variant-repeat assay [MLVA]) has been shown in various bacterial species to generate strain-specific profiles that can be compared, exchanged, and reproduced in different laboratories. The technique has been employed successfully for the typing of an increasing number of bacterial species (23). The aim of the present study was to explore the GBS genome for variably repeated genetic regions and to investigate the feasibility of an MLVA method for typing the bacterium.

MATERIALS AND METHODS

Selection, culture, and DNA extraction of GBS strains. A panel of 126 GBS strains was selected to represent a broad range of CPS and surface proteins and sequence types. Most of these (n = 113) were invasive strains submitted to the Norwegian reference laboratory for GBS. They were also used in a previous study of invasive GBS in infants (3). In addition, nine international reference strains were included, among them all eight fully sequenced strains. Two Zimbabwean colonizing strains and two Danish CPS type IX strains were also included. The distribution of CPS types was as follows: Ia, 12 strains; Ib, 10 strains; II, 6 strains; III, 55 strains; IV, 9 strains; V, 30 strains; VI, 1 strain; and IX, 3 strains. Strains stored at −80°C were grown overnight on blood agar plates. For nucleic acid extraction, 4 to 5 colonies were added to 200 μl lysis solution containing 12 μl (20 mg/ml) lysozyme (Sigma-Aldrich Corp., St. Louis, MO), 4.8 μl (20 mg/ml) proteinase K (Sigma), 4.8 μl (10,000 U/ml) mutanolysin (Sigma), and 178.4 μl Tris-EDTA (TE) buffer and incubated at 37°C and 65°C for 15 min each. DNA was purified on a Qiagen BioRobot M48 instrument using the MagAttractDNA Mini 48 kit (Qiagen, Hilden, Germany) and eluted in a volume of 50 μl. The eluate was diluted 1:10 for PCR.

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TABLE 1. Properties of 18 assumed variable genetic loci in *Streptococcus agalactiae*

Name[a]	Strain[b]	Position in strain genome	Repeat size and count in original strain (offset size)	Annotation in genome
TR1 (SATR1)	2603 V/R	143000	60 bp × 3 (139 bp)	Hypothetical protein SAG0134, CRISPR
TR2*	A909	115500	9 bp × 4	
TR3 (SATR2)	A909	746100	18 bp × 6 (85 bp)	Hypothetical protein SAK_0805
TR4*	A909	1159300	18 bp × 2	
TR5*	A909	1256100		
TR6 (SATR3)	A909	1993600	12 bp × 23 (92 bp)	Surface adhesion protein gbs2018 in NEM316
TR7*	A909	2071160		
TR8 (SATR4)	NEM316	29700	18 bp × 3 (99 bp)	*pcsB* protein
TR9	NEM316	107750	24 bp × 2	Chaperone protein, *dnaJ*
TR10*	NEM316	476780		
TR11*	NEM316	1547230		
TR12 (SATR5)	A909	1118500	48 bp × 14 (161 bp)	Putative fibrinogen-binding protein, SAK_1142
TR13	2603 V/R	451800		45-bp insert upstream of *alp* proteins
TR14	NEM316	857700		53-bp insert, gene gbs0831
TR15*	NEM316	917600		
TR16	NEM316	1010700		66-bp insert, hypothetical protein gbs0966
TR17	NEM316	1269700		45-bp deletion in A909, intergenetic between gbs1230 and *ung*
TR18	NEM316	1624650		44-bp deletion, gbs1557

[a] *, not surveyed further after initial screening.
[b] The strain column refers to the strain in which the locus was found originally.

Detection of VNTRs, PCR protocols, and analysis of fragments. Genomes were analyzed using the Tandem Repeats Finder program, version 4.00 (1). The Variable Region Finder available at the Health Protection Agency website (http://www.hpa-bioinformatics.org.uk/variable_region_finder/index.html) was also used. Primers were designed using Oligo 6.71 software (Molecular Biology Insights, Inc., Cascade, CO). The loci were consecutively termed TR1 through TR18, while the five loci proposed for the subsequent MLVA were renamed SATR1 to SATR5 in the later phase of the study. Analysis of the repeats was generally done with single PCRs in 25 μl of reaction mixture containing 50 μM (each) dATP, dCTP, dGTP, and dTTP; forward and reverse primers (0.5 μM); 2.5 μl 10× PCR buffer with 15 mM MgCl and 0.75 U AmpliTaq Gold DNA polymerase (both of the latter by Applied Biosystems, Foster City, CA); a 1-μl aliquot of the purified, diluted DNA template; and RNase-free water. The PCR conditions were 5 min at 95°C for activation of the polymerase and then 35 cycles of 30 s at 95°C, 30 s at annealing temperature, and 60 s at 72°C. The annealing temperature used initially was that proposed by the primer design software, but the temperature was later changed to 55°C. The PCR was performed on an MJ Research PTC-200 instrument (MJ Research, Inc., Watertown, MA). Amplicon size was determined with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) using the DNA 1000 chip. Later, amplicon size was determined by either 2% agarose gel electrophoresis for the VNTRs that showed only two or three alleles or by fragment analysis with capillary electrophoresis. The Qiagen multiplex PCR kit was used for the subsequent multiplex PCR. The manufacturer's recommendations were followed, except for the annealing temperature, which was set to 55°C. In addition, the concentrations of the individual primers in the primer mix were adjusted to 1.5 μM for SATR1 and -5, 1 μM for SATR2 and -3, and 0.5 μM for SATR4, and the total PCR volume was reduced to 25 μl.

Fragment analysis was done on an ABI 3130xl genetic analyzer. Hi-Di formamide, GeneScan 1200 LIZ size standard, and the PCR product were mixed (9 plus 0.5 plus 0.5 μl). The standard protocol for fragment analysis with 36-cm capillaries and POP7 polymer was used. The product sizes were analyzed using the GeneMapper 4.0 software. (All products used for fragment analysis were from Applied Biosystems.)

For analysis of the stability of the alleles, three strains (06/14, 06/123, and 07/15) were passaged on blood agar plates 40 times over a period of 4 months.

Serotyping and MLST. Typing of CPS and surface proteins was done by PCR (6, 11, 24), except that immunofluorescence microscopy was used for the nine Norwegian strains collected in 2005 (4). MLST was performed as described elsewhere (9). Results were available for all 126 of the strains. STs of most of the Norwegian strains were available from a previous study (3), whereas the ST of the international reference strains was available from a recent publication (22).

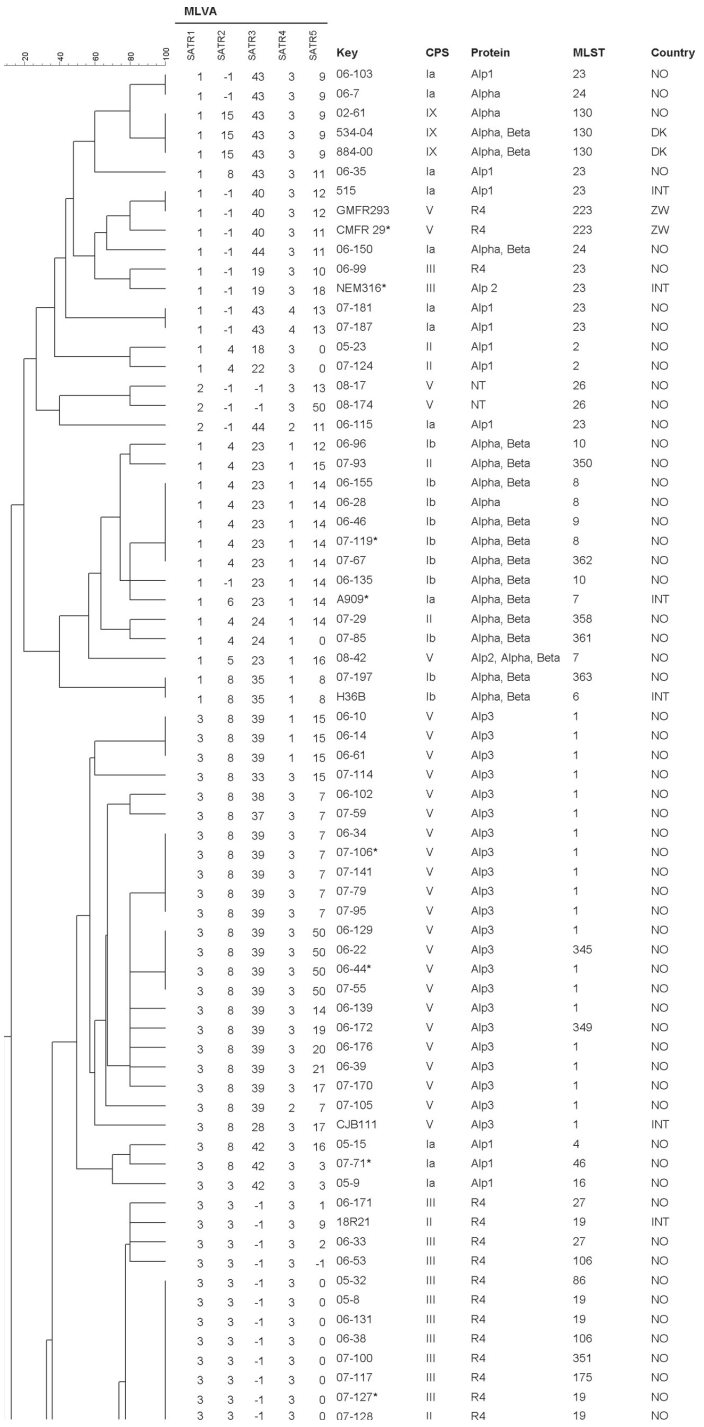
Analysis of results. For cluster analysis, BioNumerics 6.0 software (Applied Maths, Sint-Martens-Latem, Belgium) was used. Simpson's index of diversity was calculated either via the VNTR DIversity and Confidence Extractor (V-DICE) software at the Health Protection Agency website (Health Protection Agency, Colindale, London, United Kingdom; http://www.hpa-bioinfotools.org.uk/cgi-bin/DICI/DICI.pl) or manually (18). The number of repeats was determined by subtracting the offset (the number of nucleotides between the primers and the start/end of the repeat) and dividing the remaining number of nucleotides by the repeat length (Table 1).

Sequence verification. For DNA sequencing, loci were amplified in PCRs with unlabeled primers as described above. The PCR products were purified by using ExoSAP-IT (USB Corporation, Cleveland, OH) following the instructions of the manufacturer. BigDye 3.1 and BigDye terminator 3.1 (Applied Biosystems) reagents were used according to the manufacturer's instructions for sequencing PCR and subsequent purification. Capillary electrophoresis of sequencing PCR products was performed on an ABI 3130xl genetic analyzer (Applied Biosystems). The resulting sequences were analyzed using Sequencher 4.2 software (Gene Codes Corporation, Ann Arbor, MI).

RESULTS

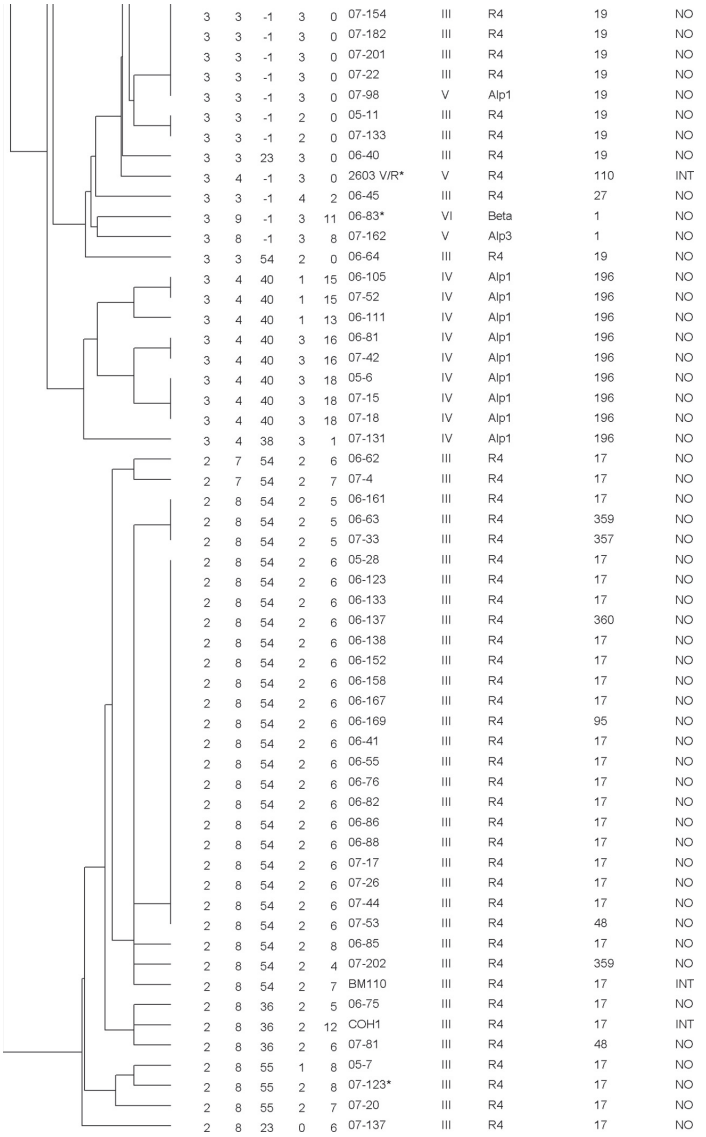
Identification of tandem repeats in GBS. The sequences of the three complete genomes of strains A909, NEM316, and 2603 V/R were analyzed *in silico* using the Tandem Repeats Finder program or the Variable Region Finder website. Candidate loci were compared via the BLAST website with the eight published whole-genome sequences, namely, the three above mentioned and five not fully assembled and annotated shotgun sequences (strains 515, H36B, 18RS21, COH1, and CJB111) (22). Most loci were excluded from further investigation for reasons such as large size, poorly conserved repeats, or poorly conserved flanking sequences. For the 18 remaining loci (Table 1), primers were designed. Their presence and their suitability for an MLVA were tested in 11 GBS strains. Seven loci (marked with asterisks in Fig. 1) were found unsuitable because they did not show variability or were not amplified even after several primer pairs were designed and tested.

The remaining 11 loci were investigated in all 126 strains. Four of the loci had four or more alleles, and three had three alleles, while the remaining four loci had two alleles in this strain collection. Six of the 11 diverse loci did not generate a PCR product in some strains (indicated as −1 repeats in Fig. 1). Amplification of SATR4 and SATR5 generated a PCR



SATR1	SATR2	SATR3	SATR4	SATR5	Key	CPS	Protein	MLST	Country
1	-1	43	3	9	06-103	Ia	Alp1	23	NO
1	-1	43	3	9	06-7	Ia	Alpha	24	NO
1	15	43	3	9	02-61	IX	Alpha	130	NO
1	15	43	3	9	534-04	IX	Alpha, Beta	130	DK
1	15	43	3	9	884-00	IX	Alpha, Beta	130	DK
1	8	43	3	11	06-35	Ia	Alp1	23	NO
1	-1	40	3	12	515	Ia	Alp1	23	INT
1	-1	40	3	12	GMFR293	V	R4	223	ZW
1	-1	40	3	11	CMFR 29*	V	R4	223	ZW
1	-1	44	3	11	06-150	Ia	Alpha, Beta	24	NO
1	-1	19	3	10	06-99	III	R4	23	NO
1	-1	19	3	18	NEM316*	III	Alp 2	23	INT
1	-1	43	4	13	07-181	Ia	Alp1	23	NO
1	-1	43	4	13	07-187	Ia	Alp1	23	NO
1	4	18	3	0	05-23	II	Alp1	2	NO
1	4	22	3	0	07-124	II	Alp1	2	NO
2	-1	-1	3	13	08-17	V	NT	26	NO
2	-1	-1	3	50	08-174	V	NT	26	NO
2	-1	44	2	11	06-115	Ia	Alp1	23	NO
1	4	23	1	12	06-96	Ib	Alpha, Beta	10	NO
1	4	23	1	15	07-93	II	Alpha, Beta	350	NO
1	4	23	1	14	06-155	Ib	Alpha, Beta	8	NO
1	4	23	1	14	06-28	Ib	Alpha	8	NO
1	4	23	1	14	06-46	Ib	Alpha, Beta	9	NO
1	4	23	1	14	07-119*	Ib	Alpha, Beta	8	NO
1	4	23	1	14	07-67	Ib	Alpha, Beta	362	NO
1	-1	23	1	14	06-135	Ib	Alpha, Beta	10	NO
1	6	23	1	14	A909*	Ia	Alpha, Beta	7	INT
1	4	24	1	14	07-29	II	Alpha, Beta	358	NO
1	4	24	1	0	07-85	Ib	Alpha, Beta	361	NO
1	5	23	1	16	08-42	V	Alp2, Alpha, Beta	7	NO
1	8	35	1	8	07-197	Ib	Alpha, Beta	363	NO
1	8	35	1	8	H36B	Ib	Alpha, Beta	6	INT
3	8	39	1	15	06-10	V	Alp3	1	NO
3	8	39	1	15	06-14	V	Alp3	1	NO
3	8	39	1	15	06-61	V	Alp3	1	NO
3	8	33	3	15	07-114	V	Alp3	1	NO
3	8	38	3	7	06-102	V	Alp3	1	NO
3	8	37	3	7	07-59	V	Alp3	1	NO
3	8	39	3	7	06-34	V	Alp3	1	NO
3	8	39	3	7	07-106*	V	Alp3	1	NO
3	8	39	3	7	07-141	V	Alp3	1	NO
3	8	39	3	7	07-79	V	Alp3	1	NO
3	8	39	3	7	07-95	V	Alp3	1	NO
3	8	39	3	50	06-129	V	Alp3	1	NO
3	8	39	3	50	06-22	V	Alp3	345	NO
3	8	39	3	50	06-44*	V	Alp3	1	NO
3	8	39	3	50	07-55	V	Alp3	1	NO
3	8	39	3	14	06-139	V	Alp3	1	NO
3	8	39	3	19	06-172	V	Alp3	349	NO
3	8	39	3	20	06-176	V	Alp3	1	NO
3	8	39	3	21	06-39	V	Alp3	1	NO
3	8	39	3	17	07-170	V	Alp3	1	NO
3	8	39	2	7	07-105	V	Alp3	1	NO
3	8	28	3	17	CJB111	V	Alp3	1	INT
3	8	42	3	16	05-15	Ia	Alp1	4	NO
3	8	42	3	3	07-71*	Ia	Alp1	46	NO
3	3	42	3	3	05-9	Ia	Alp1	16	NO
3	3	-1	3	1	06-171	III	R4	27	NO
3	3	-1	3	9	18R21	II	R4	19	INT
3	3	-1	3	2	06-33	III	R4	27	NO
3	3	-1	3	-1	06-53	III	R4	106	NO
3	3	-1	3	0	05-32	III	R4	86	NO
3	3	-1	3	0	05-8	III	R4	19	NO
3	3	-1	3	0	06-131	III	R4	19	NO
3	3	-1	3	0	06-38	III	R4	106	NO
3	3	-1	3	0	07-100	III	R4	351	NO
3	3	-1	3	0	07-117	III	R4	175	NO
3	3	-1	3	0	07-127*	III	R4	19	NO
3	3	-1	3	0	07-128	II	R4	19	NO

FIG. 1. Cluster analysis of 126 GBS strains using categorical values and the UPGMA (unweighted-pair group method using average linkages) algorithm, generated with Bionumerics 6.0 software. Numbers in the SATR1 to -5 columns indicate repeat count (−1 repeat, no PCR product). CPS, capsular polysaccharide. The country column gives the origin of the strain: NO, Norway; INT, international reference strain; DK, Denmark; and ZW, Zimbabwe. Strains marked with asterisks were used in the initial analysis of 18 variable loci. There is an overlap of three strains between the two panels.



3	3	-1	3	0	07-154	III	R4	19	NO
3	3	-1	3	0	07-182	III	R4	19	NO
3	3	-1	3	0	07-201	III	R4	19	NO
3	3	-1	3	0	07-22	III	R4	19	NO
3	3	-1	3	0	07-98	V	Alp1	19	NO
3	3	-1	2	0	05-11	III	R4	19	NO
3	3	-1	2	0	07-133	III	R4	19	NO
3	3	23	3	0	06-40	III	R4	19	NO
3	4	-1	3	0	2603 V/R*	V	R4	110	INT
3	3	-1	4	2	06-45	III	R4	27	NO
3	9	-1	3	11	06-83*	VI	Beta	1	NO
3	8	-1	3	8	07-162	V	Alp3	1	NO
3	3	54	2	0	06-64	III	R4	19	NO
3	4	40	1	15	06-105	IV	Alp1	196	NO
3	4	40	1	15	07-52	IV	Alp1	196	NO
3	4	40	1	13	06-111	IV	Alp1	196	NO
3	4	40	3	16	06-81	IV	Alp1	196	NO
3	4	40	3	16	07-42	IV	Alp1	196	NO
3	4	40	3	18	05-6	IV	Alp1	196	NO
3	4	40	3	18	07-15	IV	Alp1	196	NO
3	4	40	3	18	07-18	IV	Alp1	196	NO
3	4	38	3	1	07-131	IV	Alp1	196	NO
2	7	54	2	6	06-62	III	R4	17	NO
2	7	54	2	7	07-4	III	R4	17	NO
2	8	54	2	5	06-161	III	R4	17	NO
2	8	54	2	5	06-63	III	R4	359	NO
2	8	54	2	5	07-33	III	R4	357	NO
2	8	54	2	6	05-28	III	R4	17	NO
2	8	54	2	6	06-123	III	R4	17	NO
2	8	54	2	6	06-133	III	R4	17	NO
2	8	54	2	6	06-137	III	R4	360	NO
2	8	54	2	6	06-138	III	R4	17	NO
2	8	54	2	6	06-152	III	R4	17	NO
2	8	54	2	6	06-158	III	R4	17	NO
2	8	54	2	6	06-167	III	R4	17	NO
2	8	54	2	6	06-169	III	R4	95	NO
2	8	54	2	6	06-41	III	R4	17	NO
2	8	54	2	6	06-55	III	R4	17	NO
2	8	54	2	6	06-76	III	R4	17	NO
2	8	54	2	6	06-82	III	R4	17	NO
2	8	54	2	6	06-86	III	R4	17	NO
2	8	54	2	6	06-88	III	R4	17	NO
2	8	54	2	6	07-17	III	R4	17	NO
2	8	54	2	6	07-26	III	R4	17	NO
2	8	54	2	6	07-44	III	R4	17	NO
2	8	54	2	6	07-53	III	R4	48	NO
2	8	54	2	8	06-85	III	R4	17	NO
2	8	54	2	4	07-202	III	R4	359	NO
2	8	54	2	7	BM110	III	R4	17	INT
2	8	36	2	5	06-75	III	R4	17	NO
2	8	36	2	12	COH1	III	R4	17	INT
2	8	36	2	6	07-81	III	R4	48	NO
2	8	55	1	8	05-7	III	R4	17	NO
2	8	55	2	8	07-123*	III	R4	17	NO
2	8	55	2	7	07-20	III	R4	17	NO
2	8	23	0	6	07-137	III	R4	17	NO

FIG. 1—*Continued.*

product devoid of the repeat in 1 strain and 21 strains, respectively (indicated as 0 repeats in Fig. 1). The number of alleles for the 11 loci and their calculated diversity index are shown in Table 2. All 11 loci were stable in three strains that were passaged 40 times.

Construction of an MLVA. The five most diverse loci were chosen for the construction of a GBS MLVA with fluorescently labeled primers and size estimation by capillary electrophoresis. The five proposed MLVA loci (SATR1 to -5) were amplified in one multiplex PCR. Due to uneven amplification efficiency, the primer concentration had to be adjusted as described above. Fragment sizes were estimated on an ABI 3130xl Genetic Analyzer using the 1200 LIZ size standard. This standard was chosen because SATR3 and SATR5 had large PCR products in some strains: up to around 750 bp in SATR3 and above 1,200 bp in the latter.

The five loci are described in more detail as follows.

(i) SATR1 (TR1). This SATR1 repeat locus consisted of well-conserved repeats of 60 bp and was present in all strains investigated with one to three repeats. This locus was previ-

TABLE 2. Primers, fluorescent dyes, and Simpson's index of diversity for 11 VTNR loci in GBS

Locus	Sequence (5′→3′)[a]		Diversity index[b]	95% CI	*K*[c]	max(pi)[d]
	Forward primer	Reverse primer				
SATR5 (TR12)	6-FAM-AGTCACCTTGACTAGAGTG	AATAAAATAGGTTTTAGAACTTGG	0.913	0.903–0.923	24	0.173
SATR3 (TR6)	VIC-CCGCCAAGTTTCCGCTAG	AGCTAACAAGCTACGTGATGC	0.869	0.856–0.881	19	0.220
SATR2 (TR3)	PET-GTTGATAAAGTTGATGT TCCG	AGCCTTCTTCAACTATAGGTG	0.703	0.675–0.732	9	0.472
SATR1 (TR1)	NED-ACTTGTGAAAGTGTATCGGT	AAATCAGTGTTTTAACAGCAGC	0.643	0.626–0.661	3	0.465
SATR4 (TR8)	VIC-AAAGCATCTTTAATTCAGGCA	CTGTGGCAGACTCAACTTGTG	0.633	0.608–0.658	5	0.504
TR9	TGTCTAAGAAATACCATCCAG	TTCAAAACTTAAATTAACACG	0.470	0.444–0.496	3	0.643
TR13	TGTTTCTAAGAAAAAAGAGG	ACCTTACATTTGATTACACC	0.470	0.444–0.496	3	0.643
TR16	ATCAAAAAACCAGATACTGC	CAATGCTTTAACCGAACCAG	0.455	0.431–0.478	2	0.651
TR14	ATTATAGAGGACGTTACTCG	CAACCATCCGACCTGTAATA	0.172	0.133–0.212	2	0.905
TR18	ATGACAGTTGAACATGTGGA	TACCATGTACCAACAGACTG	0.031	0.011–0.051	2	0.984
TR17	AATTTGGTTCCGTTTGGCT	ATGCTCGAAAGAAAAAGTCG	0.016	0.001–0.030	2	0.992

[a] Certain primers were labeled with the fluorescent dyes 6-FAM (6-carboxyfluorescein), VIC, PET, and NED, as indicated.
[b] The diversity index (for VNTR data) is a measure of the variation of the number of repeats at each locus, ranging from 0.0 (no diversity) to 1.0 (complete diversity). The most diverse loci are shown at the top.
[c] *K*, number of different repeats present at this locus in a collection of 126 GBS strains.
[d] max(pi), fraction of samples that have the most frequent repeat number in this locus (range, 0.0 to 1.0).

ously described by Dmitriev et al. (7, 17) as consisting of 16-bp direct repeats and 44-bp spacer regions.

(ii) SATR2 (TR3). The SATR2 repeats were well conserved, consisting of 18 bp, and the strains contained 3 to 15 repeats. All sequenced strains had an insert of 9 bp found between repeats 2 and 3. The only strains having 15 repeats were the three strains in this study of the recently described serotype IX (20). In 14 strains (11%), no PCR product was detected.

(iii) SATR3 (TR6). The SATR3 repeat locus gave comparably large products from 297 to 750 bp. The repeat unit of 12 bp was quite well conserved. The repeat count was computed to 18 to 54 repeats; however, sequencing demonstrated that the strains with the largest amplicons had 18 repeats and an inserted sequence of two 216-bp repeats. This combination was found in 33 strains (26.4%), all but one of which belonged to MLST clonal complex 17. A similar observation was made by Lamy et al. (12), as discussed below. SATR3 was not amplified in 25 strains (20%), most of which were of serotype III/R4 and MLST clonal complex 19.

(iv) SATR4 (TR8). The SATR4 repeat gave the smallest PCR products, ranging between 99 and 168 bp. The underlying repeat of 18 bp was quite degenerated; in some strains, some or all of the repeats were only 15 bp. For convenience of typing, all alleles are assigned as multiples of 18 bp in the calculation of repeat numbers. A PCR product was observed in all 126 strains, and five different alleles were found. The repeat is located in the *pcsB* gene, encoding a protein which is important for cell wall separation (15).

(v) SATR5 (TR12). The SATR5 locus is the most diverse repeat locus in this study, with 24 different alleles in the 126 strains and a Simpson's index of diversity of 0.913 (confidence interval [CI], 0.900 to 0.922). The repeat of 48 bp was quite well conserved, and an amplification product was detected in all but one strain. Of 21 strains with a PCR product size equivalent to the offset (0 repeats), 13 were of ST19. A result of six repeats was found in 22 strains, 18 of which belonged to ST17. Some strains had an insert after the repeat locus, causing a variable offset. However, for typing purposes, the offset was set to 161 bp with the primers chosen. The larger products of this locus, especially those above 900 bp, did give weak signals in spite of attempts to optimize both multiplex PCR and fragment analysis. If SATR5 did not yield a product in the multiplex PCR, it was repeated in a single PCR. In five strains, the SATR5 locus was calculated to be more than 1,400 bp by the GeneMapper software, although this was above the upper limit of the 1200 LIZ size standard and therefore unreliable. When the five strains containing SATR5 amplicons above 1,200 bp were analyzed by gel electrophoresis, they showed diversity. In the analysis, however, these five strains were assigned a repeat count of 50. SATR5 is located in the gene encoding the fibrinogen binding protein FbsA (16).

Comparison between MLVA, serotyping, and MLST. MLVA analysis with the five proposed loci resolved the 126 strains into 70 different types with a Simpson's index of diversity of 0.963, while MLST separated them into 36 STs with an index of 0.899. The two largest groups of identical MLVA profiles consisted of 19 strains and 13 strains, most of which belonged to ST17 and ST19, respectively. These two MLST types are common subgroups of CPS type III (Fig. 1). All 27 of the ST17 strains in this work clustered together but were resolved into 12 different MLVA types within this cluster; 16 of them had identical profiles (Fig. 2). Similarly, all 11 CPS III/ST19 strains belonged to the same MLVA cluster and were resolved into four MLVA types; 7 of them had identical profiles. CPS type V has previously been described as relatively homogenous, possibly due to a recent introduction of this group as a human pathogen (5). Most of the type V strains belonged to ST1 (21 out of 30 type V strains); 20 of these 21 were in a cluster consisting of 12 MLVA types, mostly because of heterogeneity in SATR5.

A simpler MLVA analysis using SATR1 to -4 resulted in 43 different types for the 126 strains (Simpson's index of diversity, 0.926), also corresponding very well with the clustering by the other typing methods.

DISCUSSION

This study identified VNTR loci in the genome of GBS that are suitable for MLVA typing. MLVA is a relatively new typing method with several advantages compared to other methods.

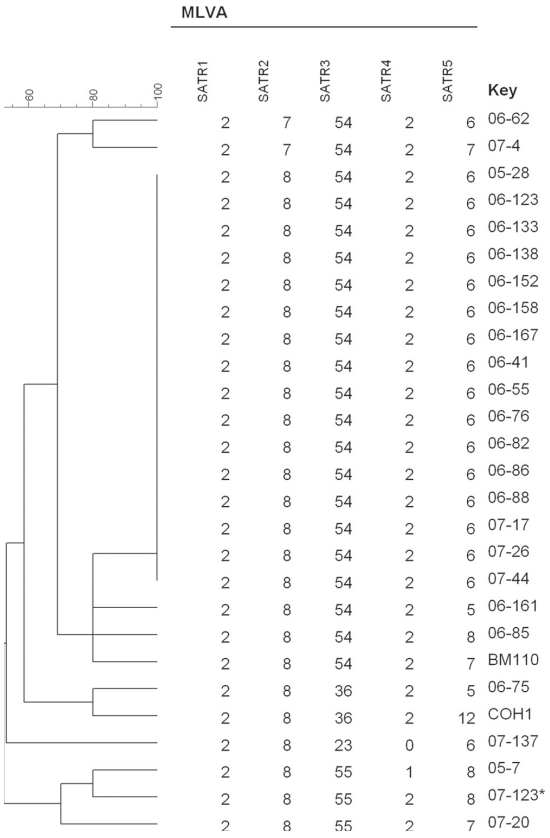


FIG. 2. MLVA cluster analysis of 27 ST17 strains included in the study using categorical values and the UPGMA algorithm, generated with Bionumerics 6.0 software. Numbers in the SATR1 to -5 columns indicate the repeat count.

Mainly, MLVA is easy to perform at moderate costs, and results are in most cases available within 2 days, which will usually be more rapid than MLST or PFGE. Since MLVA generates numeric results, comparison of strains between different laboratories may easily be done. In the present study, 18 candidate loci were identified in the GBS genome by computational studies. Initial laboratory analysis found seven of these not suitable. The remaining 11 loci were tested in 126 strains, and the five most diverse loci were selected for an MLVA based on multiplex PCR and capillary electrophoresis for determination of the number of repeats.

The strain collection analyzed consisted of Norwegian invasive GBS strains and international reference strains which together represented a wide selection of CPS types and STs. The Norwegian strains, mostly from neonates, had been submitted to the national reference laboratory from hospitals throughout the country in the years 2006 and 2007. It is therefore reasonable to assume that there was no epidemiological association between most patients. Based on the high discriminatory power of the MLVA shown in this study, the method may well be suited for outbreak investigation. This should be addressed in a study with epidemiologically related strains.

Typing with MLVA in this study did generally correspond with the clustering of strains observed in CPS/surface protein typing or MLST. The 5-locus MLVA provided a very high degree of resolution, discriminating the 126 GBS strains into 70 different MLVA types (Fig. 1). The discriminatory power of this MLVA is superior to those of both MLST and combined CPS/protein typing, which resolved the strains into 36 and 19 types, respectively. The strains could have been resolved further if SATR5 alleles above 1,200 bp had been differentiated, which could be necessary under certain circumstances. If required, this will imply the use of other methods for exact size enumeration of SATR5 and consequently a delay of results. For SATR4, the diversity due to the degenerate character of the repeat should be kept in mind. In addition to the repeat count, the fragment size may be considered, especially if the MLVA is used in epidemiological investigations. As an alternative option for providing rapid results, a 4-locus MLVA, consisting of the loci SATR1 to -4 was also analyzed. Even this 4-locus MLVA was capable of providing higher resolution than MLST or CPS/protein typing. Conversely, some or all the six loci mentioned in Table 2 but not included in the 5-locus MLVA analysis (e.g., TR9, TR13, and TR16) could be added to the MLVA if an even higher degree of resolution is desirable.

Some of the loci selected for the 5-locus MLVA have been investigated previously for other purposes. SATR1 has been addressed by a Russian and Slovakian group (7, 17) who found eight alleles in 112 bovine strains. Strictly speaking, this locus is a clustered regularly interspaced short palindromic repeat (CRISPR) but worked very well as component of this MLVA. SATR3 is located in the gene of surface adhesion protein gbs2018 previously analyzed by Lamy et al. (12). They described three alleles, one of which included the insert of 216 bp with two repeats mentioned above and found it as well strongly correlated with ST17. GBS belonging to ST17 account for a high proportion of newborn infections (3, 9, 14); however, whether these GBS represent a hypervirulent cluster is under discussion (13). SATR5 is the most diverse repeat locus and is responsible for a substantial part of the overall resolution obtained with this assay. Analyzed alone, it did cluster some of the MLST groups such as ST17 and ST19 well, while others (e.g., CPS type V/ST1) were more dispersed. On the other hand, SATR5 resolved CPS-type V/ST1 into many different types in the MLVA, while other typing techniques tend to give a much more homogeneous picture of this subgroup. In SATR2, the finding of 15 repeats was indicative for CPS type IX. This was also observed in three additional type IX strains not included in this study. Results that were indicative of certain sequence types were, e.g., the finding of 54 or 55 repeats in SATR3 for ST17 (Fig. 2). Similarly, an ST19 could be expected when SATR3 had no PCR product (−1 repeats) and SATR5 had 0 repeats.

To summarize, the present study identified VNTR loci in the genome of *Streptococcus agalactiae* suitable for MLVA analysis. Five loci were selected for a multiplex PCR protocol followed by capillary electrophoresis, enabling a very discriminatory GBS typing assay. The 5-locus MLVA resolved a strain collection of 126 GBS strains considerably better than MLST

and with less workload. This method is well suited for typing of GBS, e.g., by national reference laboratories and for research purposes. Further work with this method would include refinement of the method, comparison with PFGE with epidemiologically related strains, as well as analysis of other strain collections, such as non-Norwegian strains and noninvasive and animal strains, and establishment of a website for international collaboration and strain comparison.

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Paper III

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Multiple-locus variant-repeat assay (MLVA) is a useful tool for molecular epidemiologic analysis of *Streptococcus agalactiae* strains causing bovine mastitis

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ABSTRACT

Group B streptococci (GBS) were considered a major cause of mastitis in cattle until preventive measures succeeded in controlling the disease in the 1970s and 1980s. During the last 5–6 years an increasing number of cases have been observed in some Scandinavian countries. A total of 187 GBS isolates from mastitis cases were collected from 119 animals in 34 Norwegian farms in the period from April 2007 to November 2010. 133 (71%) of the isolates were from farms with automated milking systems. The strains underwent typing of capsular polysaccharides (CPS) and surface proteins, and were analyzed by multi-locus variable repeat assay (MLVA) to investigate the epidemiological relationship of strains within and between farms. The GBS strains were differentiated into 12 types by CPS and surface protein analysis, with CPS types V (54%) and IV (34%) predominating. MLVA was superior to CPS and protein typing for strain differentiation, resolving the 187 strains into 37 types. In 29 of 34 farms all GBS strains had identical MLVA profiles specific for each farm. However, in one farm represented with 48 isolates, four MLVA variants with differences in one repeat locus were observed during the almost 3-year long collection period. Similar variations were observed at four other farms. This might reflect the stability of repeat loci under in vivo conditions. Farms with automated milking systems were overrepresented in this material. In conclusion, the five-loci MLVA allowed rapid high-resolution genotyping of the bovine GBS strains within and between farms.

1. Introduction

Bovine mastitis caused by *Streptococcus agalactiae* (group B streptococci, GBS) is a serious problem to animal health and farm profitability. GBS mastitis can either be an acute or subclinical disease, the latter leading to gradually diminishing milk production and risk of infecting other members of the herd. Since the 1960s eradication programs have been used to control this obligate udder pathogen and succeeded in reducing the incidence of GBS mastitis (Keefe, 1997; McDonald, 1977). Before year 2000 the prevalence of infected herds was less than 2% in Scandinavia. Since then a reemergence of GBS mastitis has been observed in Scandinavia; a prevalence rate of 5.8% was reported for Denmark in 2008 (Katholm, 2010a,b; Zadoks et al., 2011). In Norway the Norwegian Veterinary Institute (NVI) observed an increased frequency of GBS positive milk samples from 0.09% in 2002 to 1.49% in 2010. The total number of milk samples analyzed was about 16,000 per year and constant during the decade (Ståle Sviland, NVI, personal communication).

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Basic typing of GBS usually determines the capsular polysaccharide (CPS) type. This can be done by immunologic methods, but many bovine strains have been found to be non-typable (Jensen and Aarestrup, 1996; Zhao et al., 2006). Genotyping studies using PCR, however, demonstrated the genes for CPS in almost all bovine isolates (Sørensen et al., 2010; Zhao et al., 2006). Surface protein typing adds information to the basic typing. Newer molecular methods such as pulsed-field gel electrophoresis (Baseggio et al., 1997; Merl et al., 2003), randomly amplified polymorphic DNA analysis (Martinez et al., 2000; Pereira et al., 2010) and multi-locus sequence typing (Bisharat et al., 2004) are considerably more discriminative.

Recently we described a multi-locus variant-repeat assays (MLVA) for GBS using five variable number of tandem repeat (VNTR) loci (Radtke et al., 2010). This MLVA was more discriminatory than CPS and protein typing or multi-locus sequence typing. The increased occurrence of GBS-related mastitis in Norway prompted us to investigate the applicability of MLVA in elucidating epidemiological relationships.

2. Materials and methods

2.1. Selection, culture and DNA extraction of GBS strains

Milk samples from individual udder quarters were submitted to the Norwegian Veterinary Institute (NVI) for diagnostic bacteriology when considered indicated by the referring veterinarian or farmer. GBS isolates identified by the NVI laboratory in Trondheim between April 2007 and November 2010 were stored at −80 °C. The other local NVI laboratories (Oslo, Sandnes, Bergen and Harstad) and the Tine Norwegian Dairies BA, Mastitis Laboratory in Molde were asked for GBS isolates collected in the same period. A total of 187 GBS-isolates (148 from Central Norway, 39 from other parts of Norway) were available for analysis. The 187 samples were collected from 34 farms; 14 farms were represented by one isolate, 10 farms by 2–3 isolates and further 10 farms had four or more isolates (range 4–48). An overview of herd sizes is given in Table 1. For 181 strains isolated from 119 animals, information about the infected cow was available. Of the 119 cows, 73 were represented with one sample, 35 with two, eight with three, two with four and one with six samples. In the 46 animals that had more than one sample available, these were either taken on the same day but from different teats or taken on different occasions. Strains were cultured and subcultured on blood agar plates containing 5% ox blood at 35 °C in 5% CO_2. Fermentation of lactose was examined in phenol red broth base supplemented with 0.3% meat extract and 1% lactose (Sørensen et al., 2010). Tubes were incubated for 2 days at 35 °C. Bacitracin resistance was recorded after overnight culture on blood agar plates using bacitracin impregnated BBL Taxo Discs (Becton, Dickinson and Company, Sparks, MD, USA). For nucleic acid extraction, one colony was added to 200 μl lysis solution containing 100 μl lysozyme (Sigma-Aldrich Corp., St. Louis, MO, USA; 20 mg/ml) and 100 μl TE buffer, and was incubated at 37 °C for 15 min. DNA was purified from this lysate on a Qiagen BioRobot M48 instrument using MagAttract DNA Mini 48 Kit (Qiagen, Hilden, Germany) and eluated in a volume of 100 μl.

Table 1
Herd size of 33 farms of 34 included in the study, data for one farm (Farm ID: NS) were not available. Mean number of animals per farm and range are calculated for all 33 farms, the farms with automated milking systems (AMS) and the non-AMS farms, respectively.

	Mean	Range
All farms ($n = 33$)	39.9	7–93
AMS farms ($n = 21$)	61.4	46–93
Non-AMS farms ($n = 12$)	27.7	7–82

2.2. Detection of VNTRs, PCR protocols and analysis of fragments

Typing methods for the detection of genes encoding CPS types Ia, Ib and II through IX and surface proteins Cα, Alp1, Alp2/3, Alp4, R4 and Cβ were used as described elsewhere (Creti et al., 2004; Imperi et al., 2009; Zeng et al., 2006).

A five-loci MLVA was used as described previously (Radtke et al., 2010). Briefly, loci SATR1–4 were amplified in a multiplex-PCR with fluorescence labeled primers using the Qiagen Multiplex PCR kit (Qiagen). The manufacturer's recommendation was followed except for the annealing temperature which was set to 55 °C, and the concentrations of the individual primers in the primer mix were adjusted to 2 μM for SATR1 and 1 μM for SATR2, 3 and 4, and the total PCR volume was reduced to 25 μl. SATR5 was amplified in a separate PCR as described previously (Radtke et al., 2010), using the same PCR conditions as for the multiplex-PCR. PCRs were performed on a MJ Research PTC-200 instrument (MJ Research, Inc., Watertown, MA, USA).

Amplicon size was determined by fragment analysis with capillary electrophoresis on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The products from the two PCR reactions were both diluted 1:10 in water and mixed afterwards. Then 9 μl Hi-Di Formamide, 0.5 μl GeneScan™ 1200 LIZ Size Standard and 0.5 μl of the mixed, diluted PCR products were blended. The standard protocol for fragment analysis with 36 cm capillaries, POP7 polymer and the LIZ1200 standard was used. The product sizes were analyzed using the GeneMapper 4.0 software (all products used for fragment analysis supplied by Applied Biosystems). In six strains the SATR5 amplicon was larger than 1200 bp, which is the upper limit of the size standard. For these strains, a repeat count of 50 was assigned.

PCR for *scp*B was performed with previously published primers (Dmitriev et al., 2004) under the following conditions: 5 min at 95 °C, followed by 35 cycles of steps of 95 °C, 55 °C and 72 °C at 30 s each, and a final elongation step of 72 °C for 10 min. The PCR-products were analyzed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) using the DNA 1000 chip.

2.3. Cluster analysis

The BioNumerics 6.1 software (Applied Maths, Sint-Martens-Latem, Belgium) was used for cluster analysis,

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using categorical values and the UPGMA (unweighted-pair group method using arithmetic means) algorithm.

3. Results

In this study 187 bovine GBS strains were investigated by genotyping of capsular polysaccharides, surface proteins and MLVA. Typing of the polysaccharide capsule yielded the following results: Ia (seven strains, 3.7%), Ib (six strains, 3.2%), II (three strains, 1.6%), III (three strains, 1.6%), IV (64 strains, 34.2%), V (101 strains, 54.0%), IX (one strain, 0.5%) and two strains (1.1%) were non-typable. CPS type V and type IV together accounted for 88% of the 187 strains. CPS type V strains represented an even higher proportion of strains from Central Norway where 94 (63.5%) of 148 strains were CPS type V. From the farm (Farm ID: SS) where the two NT strains were isolated, two further strains (different cows, same timeframe) were found to be CPS-type IV. All four strains had an identical MLVA profile which may reflect a mutation in the CPS coding genes.

Genes for five of the six strain variable alpha-like proteins were found except for the rarely encountered Alp4; additionally the gene for Cβ was detected. The combination of CPS and protein typing resulted in twelve different types (Table 2).

The 187 strains were differentiated into 37 MLVA profiles (Fig. 1). In 29 of 34 farms all GBS isolates on each farm had identical MLVA profiles, different from strains from other farms. Four MLVA profiles, however, were found in more than one farm (Fig. 1). For three of these profiles the respective farms were located in different counties and some of the samples had been collected at different times. The occurrence of the fourth MLVA profile was explained by the cooperation of the involved farms (see below). On five farms more than one MLVA profile was encountered. Usually one profile predominated while a single strain with a divergent repeat number in one locus was observed (Table 3). On one farm (Farm ID: RSA) represented by two animals, the two strains encountered were different in two loci. Such in-farm polymorphisms were detected in all the five VNTR loci surveyed here. In 42 of 46 animals from which more than one sample was available, the samples for each animal had identical MLVA profiles. All four animals from which different MLVA profiles were recovered, belonged to the same farm discussed below, and the samples were taken on different occasions.

Table 2
Distribution of capsular polysaccharide (CPS) and surface protein types among 187 bovine GBS strains from 34 Norwegian farms. The number of MLVA types and number of farms per CPS/surface protein type are also given.

CPS	Protein	No. of strains	No. of MLVA types	No. of farms
Ia	Alp2/3	2	2	1
	Alp1	1	1	1
	Non-typable	4	2	2
Ib	Cα, Cβ	6	1	1
II	Alp2/3	2	1	1
	Cα, Cβ	1	1	1
III	Alp2/3	3	3	3
IV	Alp1	64	10	10
V	R4, Cβ	1	1	1
	Alp2/3	100	14	13
IX	Cα, Cβ	1	1	1
Non-typable	Alp1	2[a]	(1)	(1)
Sum		187	37	34

[a] These two strains had an identical MLVA profile as two type IV, Alp1 strain. All four strains were from the same farm.

Automated milking systems (AMS) were used on 12 (35%) of the 34 farms. Herd size was known for all but one of the farms with an average size of 39.9 animals (Table 1). Farms that had invested in AMS had bigger herd sizes (61.4 versus 27.7 cows). Of the 187 strains, 133 (71%) were collected on farms with AMS, with 0.18 isolates per cow compared to 0.09 from non-AMS farms. On average, 11 strains were available from farms with AMS compared to 2.5 strains from farms with conventional milking.

One farm (Farm ID: EH) was represented with 48 isolates from 22 cows, collected between December 2007 and September 2010 (Fig. 2). Four different MLVA profiles were found among these strains with polymorphisms occurring in SATR5 of 13, 15, 17 and 50 repeats, respectively. Two profiles predominated; in the first part of the observation period the SATR5 allele with 17 repeats occurred in 19 samples, until from September 2009 onwards an allele with 13 repeats predominated (detected in 27 strains). The other two SATR5 alleles were found only in one animal each (15 and 50 repeats); the latter was first infected with a 50 repeats strain and 2 month later with the predominant 17 repeats strain. (The strain arbitrarily sized to 50 repeats had an estimated PCR product size that would translate to 23 repeats, but the product size exceeded the size standard used.) The other animal had an infection with the 15 repeats strain and one and a half year later with the then predominant 13 repeats strain (Fig. 1). Further two cows were infected by the 17 repeats strain and later with the 13 repeats strain in a subsequent episode. The time interval between their two infections was 18 and 9 months, respectively.

One cluster of 23 samples had an identical MLVA profile in 22 samples and a single locus variant in SATR3 in the last. It originated from three farms located in the same municipality. In Farm NS several cases of GBS mastitis were diagnosed in the autumn of 2007. The farmer planned to merge his farm with another, which had not encountered problems with GBS. In preparation of this all infected cattle were treated, retested and culled if they remained infected. Thereafter an extended waiting period of almost 2 years free of acute mastitis passed before the two farms merged. After the two had merged (Farm ID: MSM) however, the problem with GBS infections reemerged and spread to another cooperating farm (Farm ID: AS). This farm had several cases of mastitis but only one GBS strain was available for this study.

Seven of the bovine MLVA profiles were also encountered in our recent study on human GBS strains (Radtke et al., 2010). In a sub-analysis the relatedness of bovine and human strains was analyzed further. 47 of the bovine isolates (representing at least one strain per farm and MLVA profile) and seven human strains were examined for lactose fermentation, bacitracin sensitivity, and presence

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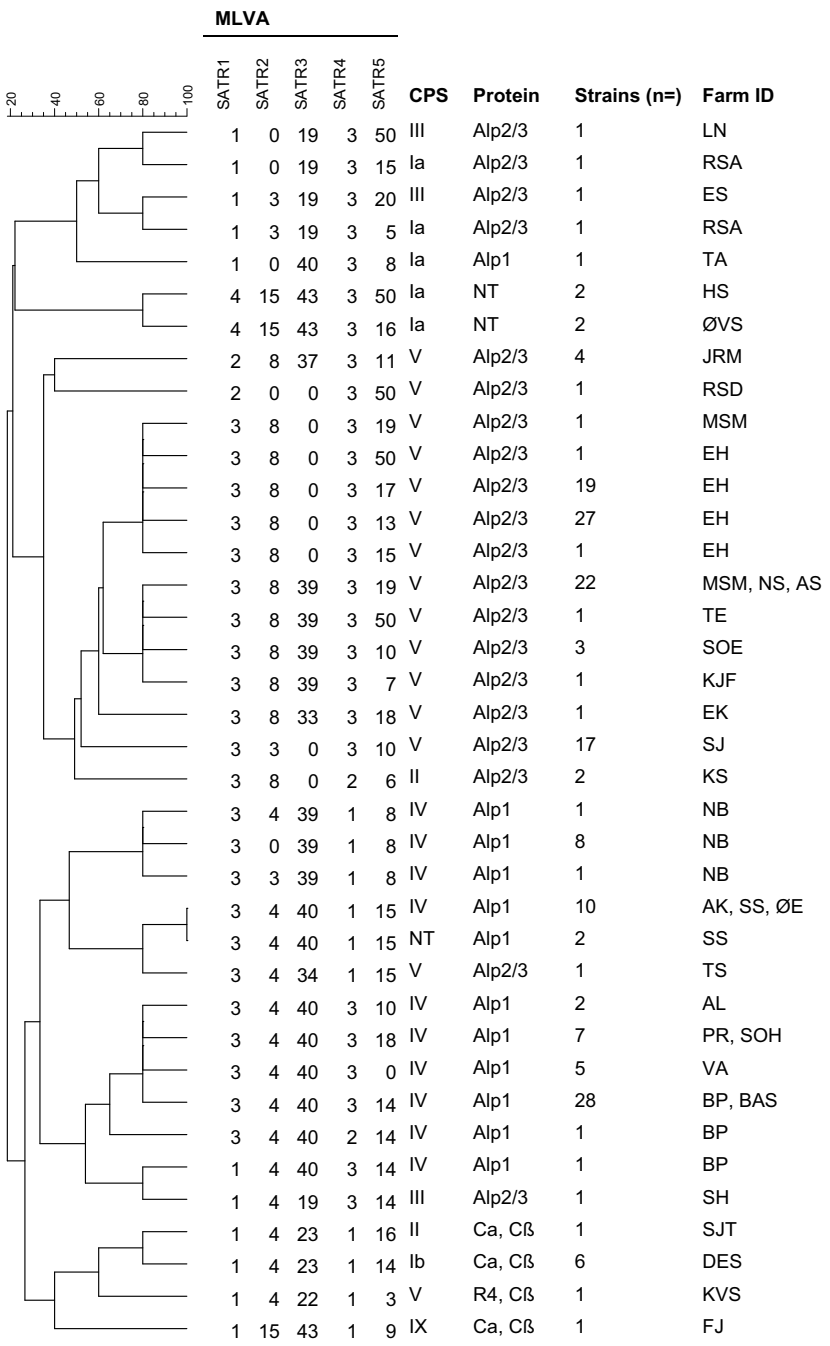

SATR1	SATR2	SATR3	SATR4	SATR5	CPS	Protein	Strains (n=)	Farm ID
1	0	19	3	50	III	Alp2/3	1	LN
1	0	19	3	15	Ia	Alp2/3	1	RSA
1	3	19	3	20	III	Alp2/3	1	ES
1	3	19	3	5	Ia	Alp2/3	1	RSA
1	0	40	3	8	Ia	Alp1	1	TA
4	15	43	3	50	Ia	NT	2	HS
4	15	43	3	16	Ia	NT	2	ØVS
2	8	37	3	11	V	Alp2/3	4	JRM
2	0	0	3	50	V	Alp2/3	1	RSD
3	8	0	3	19	V	Alp2/3	1	MSM
3	8	0	3	50	V	Alp2/3	1	EH
3	8	0	3	17	V	Alp2/3	19	EH
3	8	0	3	13	V	Alp2/3	27	EH
3	8	0	3	15	V	Alp2/3	1	EH
3	8	39	3	19	V	Alp2/3	22	MSM, NS, AS
3	8	39	3	50	V	Alp2/3	1	TE
3	8	39	3	10	V	Alp2/3	3	SOE
3	8	39	3	7	V	Alp2/3	1	KJF
3	8	33	3	18	V	Alp2/3	1	EK
3	3	0	3	10	V	Alp2/3	17	SJ
3	8	0	2	6	II	Alp2/3	2	KS
3	4	39	1	8	IV	Alp1	1	NB
3	0	39	1	8	IV	Alp1	8	NB
3	3	39	1	8	IV	Alp1	1	NB
3	4	40	1	15	IV	Alp1	10	AK, SS, ØE
3	4	40	1	15	NT	Alp1	2	SS
3	4	34	1	15	V	Alp2/3	1	TS
3	4	40	3	10	IV	Alp1	2	AL
3	4	40	3	18	IV	Alp1	7	PR, SOH
3	4	40	3	0	IV	Alp1	5	VA
3	4	40	3	14	IV	Alp1	28	BP, BAS
3	4	40	2	14	IV	Alp1	1	BP
1	4	40	3	14	IV	Alp1	1	BP
1	4	19	3	14	III	Alp2/3	1	SH
1	4	23	1	16	II	Ca, Cß	1	SJT
1	4	23	1	14	Ib	Ca, Cß	6	DES
1	4	22	1	3	V	R4, Cß	1	KVS
1	15	43	1	9	IX	Ca, Cß	1	FJ

Fig. 1. Cluster analysis of 187 bovine group B streptococci strains using categorical values and the UPGMA (unweighted-pair group method using average likages) algorithm, generated with Bionumerics 6.1 software. Numbers in the SATR1–5 columns indicate repeat count. CPS, capsular polysaccharide; Protein, surface protein; Strains (*n*=), number of strains in cluster; Farm ID, unique ID of each farm; NT, non-typable. CPS and surface proteins were inferred from the gene content investigated by PCR.

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Table 3
Five farms on which more than one MLVA type was found. MLVA profile (SATR1–5), number of cows and number of samples are given. In all cases a mutation in the predominating MLVA profile is encountered. Alterations are found in one or two (Farm ID: RSA) loci. Farm EH with 48 isolates is discussed in more detail in Section 3.

Farm ID	MLVA profile (repeats)	Cows (*n*)	Samples (*n*)
EH	3-8-0-3-17	11	19
	3-8-0-3-15	1	1
	3-8-0-3-50	1	1
	3-8-0-3-13	13	27
PB	3-4-40-3-14	12	18
	1-4-40-3-14	1	1
	3-4-40-2-14	1	1
M-SM	3-8-39-3-19	13	18
	3-8-0-3-19	1	1
NB	3-0-39-1-8	6	8
	3-3-39-1-8	1	1
	3-4-39-1-8	1	1
RSA	1-0-19-3-15	1	1
	1-3-19-3-5	1	1

of the *scp*B gene encoding C5a peptidase. All three tests had previously been used to distinguish between bovine and human strains (Finch and Martin, 1984; Sørensen et al., 2010). All but one of the bovine strains fermented lactose, while none of the seven human strains did. The only lactose negative bovine strain did not have a MLVA profile encountered among the human strains. All strains, both bovine and human strains, were resistant to bacitracin. Gene content for the C5a peptidase (*scp*B) is common in human strains. All seven human strains contained the *scp*B gene, as did 35 (74.5%) of the 47 animal strains.

4. Discussion

The current study of 187 bovine GBS strains from 34 farms showed that the MLVA method was capable of discriminating the strains into 37 genotypes. MLVA as a method for typing of GBS was found equally applicable on bovine as on human strains, a finding supported by a recently published second GBS-MLVA scheme which included both human and bovine strains (Haguenoer et al., 2011). The discrimination in our study was achieved despite the close relatedness of the majority of the strains judged from their CPS and surface protein profiles. As a rule a herd was infected by one specific MLVA type not encountered in other herds. MLVA profiles found within one herd were usually identical for all animals in that herd, with a possibility for single-locus variations in single animals (Table 3). An exception was a farm represented by only two samples with variations in two loci. One might speculate if these two loci variants are the progenies of a hypothetical common ancestor. Another interesting exception was the farm represented with 48 samples, which had a switch of genotype during the collection period, indicating that mutations could establish themselves throughout a herd (Fig. 2). One may assume that two single locus variants of the outbreak strain with 13 and 17 repeats in SATR5 predominated on this farm, while two other single locus variants with 15 and 50 repeats where detected in only one isolate each. This indicates that herds with persisting problems caused by GBS infections could be suitable for observation of the mutation pattern for different VNTR loci in vivo. Such information would be valuable to increase the quality of MLVA cluster analysis (Pourcel and Vergnaud, 2011; van Belkum et al., 2007). In our recent study on human GBS strains using MLVA, three strains were passaged 40 times in vitro without observing mutations. In this study of bovine strains with collecting periods of up to 3 years, in-farm variations in repeat numbers were detected in all five MLVA loci, demonstrating some degree of instability of these repeated genetic loci. By analogy to MLST, the term single locus variants (SLV) might also be applicable to epidemiologically related isolates differing at one locus only.

CPS encoding genes were demonstrated in all but two strains of this bovine GBS collection. Other studies using genotyping also found the genes for CPS in almost all strains (Sørensen et al., 2010; Zhao et al., 2006). In these two reports CPS-types Ia, II and III were predominant. Many veterinarian reports have used immunological methods for CPS-typing resulting in many non-typable strains, often above 50% (Martinez et al., 2000). Subsequent genotyping of the strain collection used by Martinez et al. found the gene content for CPS in 99% of the strains (Zhao et al., 2006). Merl et al. (2003) could not type CPS in 52 of 79 bovine strains. A Norwegian study from 1981, before the emergence of CPS-type V, found that type III, Ic and II were predominant, and that at least 20% of the bovine

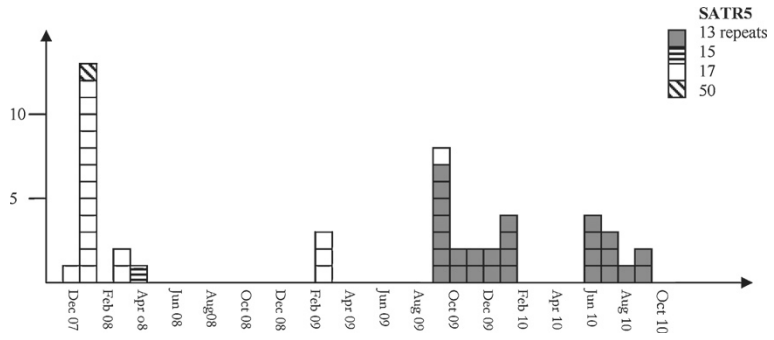

Fig. 2. Number of samples collected per month and the SATR5 repeat count for each sample are shown for a farm (Farm ID: EH) with 48 GBS samples collected over almost 3 years. Note the shift of the predominating type in SATR5 around September 2009.

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strains were non-typable (Haug et al., 1981). In a Danish collection of bovine strains from 1996 serotype III was the most prevalent, and 12 of 45 strains were non-typable using immunological methods (Jensen and Aarestrup, 1996; Katholm and Rattenborg, 2009). CPS antibodies are usually raised after immunization with human GBS strains. This might, at least in part, explain the high proportion of non-typable bovine strains by serotyping.

Capsular polysaccharide type V (54% of samples) and IV (34%) were overrepresented in our study compared to other publications. In cattle, type V has to our knowledge not been described as an important and predominant source for mastitis. Neither type IV has been reported as a frequent cause of mastitis. Type V has been encountered in increasing numbers in human invasive disease since it was first described in the early 1990s (Blumberg et al., 1996). It has established itself as the most common serotype of invasive GBS disease in adults in Norway (Bergseng et al., 2008). Lately, serotype IV has come into focus as an emerging pathogen in humans (Diedrick et al., 2010; Kiely et al., 2011), a development that has also been observed in Norway (own data, not shown). Based on the emergence of types IV and V in humans one could speculate about human to cattle transmission as a possible route of introducing GBS into herds. Systematic research on this important topic is scarce (Manning et al., 2010), and relevant studies tend to conclude that bovine and human strains are distinct entities (Richards et al., 2011; Sørensen et al., 2010).

Herds in Norway tend to be isolated on their farm, largely without immediate contact with other herds. Insemination is usually done artificially and trading of milking cows is very limited. Some contact among neighboring herds can occur during summer grazing. Transmission of GBS is therefore not very likely between farms. The finding that almost every farm in this study had its specific MLVA profile might support this.

On several of the farms with multiple samples, strains with identical or single locus variant profiles were encountered over a period of several years, indicating subclinical infection and transmission between the animals of a herd, well known traits of GBS mastitis. Farms with automated milking systems were overrepresented and did provide 0.18 GBS isolates per animal against 0.09 in farms with conventional milking. This could either be explained by a more professional approach to infections, making AMS farms more prone to order a microbiological analysis. However, an alternative explanation could be a problem associated with AMS machines. Data from the current study are not suited for a conclusive comparison of GBS prevalence between AMS and conventional dairy farms, due to the retrospective nature with possibly biased sample collection. Prospective, controlled studies addressing this subject are warranted.

In conclusion, this five-loci MLVA can be regarded as a tool well suited for rapid, highly discriminative genotyping of bovine GBS isolates, and was found to be equally applicable to bovine GBS as to human GBS strains. All but one farm had identical or single locus variant MLVA genotypes found in all animals, which was distinct from MLVA profiles from other farms. The increased occurrence of GBS in Norwegian cattle reported in this study might in part be connected to newer concepts and technology in the dairy industry, and the old principles for GBS infection control should be brought to mind again.

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180. Odrun Arna Gederaas: BIOLOGICAL MECHANISMS INVOLVED IN 5-AMINOLEVULINIC ACID BASED PHOTODYNAMIC THERAPY
181. Pål Richard Romundstad: CANCER INCIDENCE AMONG NORWEGIAN ALUMINIUM WORKERS
182. Henrik Hjorth-Hansen: NOVEL CYTOKINES IN GROWTH CONTROL AND BONE DISEASE OF MULTIPLE MYELOMA
183. Gunnar Morken: SEASONAL VARIATION OF HUMAN MOOD AND BEHAVIOUR
184. Bjørn Olav Haugen: MEASUREMENT OF CARDIAC OUTPUT AND STUDIES OF VELOCITY PROFILES IN AORTIC AND MITRAL FLOW USING TWO- AND THREE-DIMENSIONAL COLOUR FLOW IMAGING
185. Geir Bråthen: THE CLASSIFICATION AND CLINICAL DIAGNOSIS OF ALCOHOL-RELATED SEIZURES
186. Knut Ivar Aasarød: RENAL INVOLVEMENT IN INFLAMMATORY RHEUMATIC DISEASE. A Study of Renal Disease in Wegener's Granulomatosis and in Primary Sjögren's Syndrome
187. Trude Helen Flo: RESEPTORS INVOLVED IN CELL ACTIVATION BY DEFINED URONIC ACID POLYMERS AND BACTERIAL COMPONENTS
188. Bodil Kavli: HUMAN URACIL-DNA GLYCOSYLASES FROM THE UNG GENE: STRUCTRUAL BASIS FOR SUBSTRATE SPECIFICITY AND REPAIR
189. Liv Thommesen: MOLECULAR MECHANISMS INVOLVED IN TNF- AND GASTRIN-MEDIATED GENE REGULATION
190. Turid Lingaas Holmen: SMOKING AND HEALTH IN ADOLESCENCE; THE NORD-TRØNDELAG HEALTH STUDY, 1995-97
191. Øyvind Hjertner: MULTIPLE MYELOMA: INTERACTIONS BETWEEN MALIGNANT PLASMA CELLS AND THE BONE MICROENVIRONMENT

192. Asbjørn Støylen: STRAIN RATE IMAGING OF THE LEFT VENTRICLE BY ULTRASOUND. FEASIBILITY, CLINICAL VALIDATION AND PHYSIOLOGICAL ASPECTS
193. Kristian Midthjell: DIABETES IN ADULTS IN NORD-TRØNDELAG. PUBLIC HEALTH ASPECTS OF DIABETES MELLITUS IN A LARGE, NON-SELECTED NORWEGIAN POPULATION.
194. Guanglin Cui: FUNCTIONAL ASPECTS OF THE ECL CELL IN RODENTS
195. Ulrik Wisløff: CARDIAC EFFECTS OF AEROBIC ENDURANCE TRAINING: HYPERTROPHY, CONTRACTILITY AND CALCUIM HANDLING IN NORMAL AND FAILING HEART
196. Øyvind Halaas: MECHANISMS OF IMMUNOMODULATION AND CELL-MEDIATED CYTOTOXICITY INDUCED BY BACTERIAL PRODUCTS
197. Tore Amundsen: PERFUSION MR IMAGING IN THE DIAGNOSIS OF PULMONARY EMBOLISM
198. Nanna Kurtze: THE SIGNIFICANCE OF ANXIETY AND DEPRESSION IN FATIQUE AND PATTERNS OF PAIN AMONG INDIVIDUALS DIAGNOSED WITH FIBROMYALGIA: RELATIONS WITH QUALITY OF LIFE, FUNCTIONAL DISABILITY, LIFESTYLE, EMPLOYMENT STATUS, CO-MORBIDITY AND GENDER
199. Tom Ivar Lund Nilsen: PROSPECTIVE STUDIES OF CANCER RISK IN NORD-TRØNDELAG: THE HUNT STUDY. Associations with anthropometric, socioeconomic, and lifestyle risk factors
200. Asta Kristine Håberg: A NEW APPROACH TO THE STUDY OF MIDDLE CEREBRAL ARTERY OCCLUSION IN THE RAT USING MAGNETIC RESONANCE TECHNIQUES

2002

201. Knut Jørgen Arntzen: PREGNANCY AND CYTOKINES
202. Henrik Døllner: INFLAMMATORY MEDIATORS IN PERINATAL INFECTIONS
203. Asta Bye: LOW FAT, LOW LACTOSE DIET USED AS PROPHYLACTIC TREATMENT OF ACUTE INTESTINAL REACTIONS DURING PELVIC RADIOTHERAPY. A PROSPECTIVE RANDOMISED STUDY.
204. Sylvester Moyo: STUDIES ON STREPTOCOCCUS AGALACTIAE (GROUP B STREPTOCOCCUS) SURFACE-ANCHORED MARKERS WITH EMPHASIS ON STRAINS AND HUMAN SERA FROM ZIMBABWE.
205. Knut Hagen: HEAD-HUNT: THE EPIDEMIOLOGY OF HEADACHE IN NORD-TRØNDELAG
206. Li Lixin: ON THE REGULATION AND ROLE OF UNCOUPLING PROTEIN-2 IN INSULIN PRODUCING ß-CELLS
207. Anne Hildur Henriksen: SYMPTOMS OF ALLERGY AND ASTHMA VERSUS MARKERS OF LOWER AIRWAY INFLAMMATION AMONG ADOLESCENTS
208. Egil Andreas Fors: NON-MALIGNANT PAIN IN RELATION TO PSYCHOLOGICAL AND ENVIRONTENTAL FACTORS. EXPERIENTAL AND CLINICAL STUDES OF PAIN WITH FOCUS ON FIBROMYALGIA
209. Pål Klepstad: MORPHINE FOR CANCER PAIN
210. Ingunn Bakke: MECHANISMS AND CONSEQUENCES OF PEROXISOME PROLIFERATOR-INDUCED HYPERFUNCTION OF THE RAT GASTRIN PRODUCING CELL
211. Ingrid Susann Gribbestad: MAGNETIC RESONANCE IMAGING AND SPECTROSCOPY OF BREAST CANCER
212. Rønnaug Astri Ødegård: PREECLAMPSIA – MATERNAL RISK FACTORS AND FETAL GROWTH
213. Johan Haux: STUDIES ON CYTOTOXICITY INDUCED BY HUMAN NATURAL KILLER CELLS AND DIGITOXIN
214. Turid Suzanne Berg-Nielsen: PARENTING PRACTICES AND MENTALLY DISORDERED ADOLESCENTS
215. Astrid Rydning: BLOOD FLOW AS A PROTECTIVE FACTOR FOR THE STOMACH MUCOSA. AN EXPERIMENTAL STUDY ON THE ROLE OF MAST CELLS AND SENSORY AFFERENT NEURONS

2003

216. Jan Pål Loennechen: HEART FAILURE AFTER MYOCARDIAL INFARCTION. Regional Differences, Myocyte Function, Gene Expression, and Response to Cariporide, Losartan, and Exercise Training.

217. Elisabeth Qvigstad: EFFECTS OF FATTY ACIDS AND OVER-STIMULATION ON INSULIN SECRETION IN MAN
218. Arne Åsberg: EPIDEMIOLOGICAL STUDIES IN HEREDITARY HEMOCHROMATOSIS: PREVALENCE, MORBIDITY AND BENEFIT OF SCREENING.
219. Johan Fredrik Skomsvoll: REPRODUCTIVE OUTCOME IN WOMEN WITH RHEUMATIC DISEASE. A population registry based study of the effects of inflammatory rheumatic disease and connective tissue disease on reproductive outcome in Norwegian women in 1967-1995.
220. Siv Mørkved: URINARY INCONTINENCE DURING PREGNANCY AND AFTER DELIVERY: EFFECT OF PELVIC FLOOR MUSCLE TRAINING IN PREVENTION AND TREATMENT
221. Marit S. Jordhøy: THE IMPACT OF COMPREHENSIVE PALLIATIVE CARE
222. Tom Christian Martinsen: HYPERGASTRINEMIA AND HYPOACIDITY IN RODENTS – CAUSES AND CONSEQUENCES
223. Solveig Tingulstad: CENTRALIZATION OF PRIMARY SURGERY FOR OVARAIN CANCER. FEASIBILITY AND IMPACT ON SURVIVAL
224. Haytham Eloqayli: METABOLIC CHANGES IN THE BRAIN CAUSED BY EPILEPTIC SEIZURES
225. Torunn Bruland: STUDIES OF EARLY RETROVIRUS-HOST INTERACTIONS – VIRAL DETERMINANTS FOR PATHOGENESIS AND THE INFLUENCE OF SEX ON THE SUSCEPTIBILITY TO FRIEND MURINE LEUKAEMIA VIRUS INFECTION
226. Torstein Hole: DOPPLER ECHOCARDIOGRAPHIC EVALUATION OF LEFT VENTRICULAR FUNCTION IN PATIENTS WITH ACUTE MYOCARDIAL INFARCTION
227. Vibeke Nossum: THE EFFECT OF VASCULAR BUBBLES ON ENDOTHELIAL FUNCTION
228. Sigurd Fasting: ROUTINE BASED RECORDING OF ADVERSE EVENTS DURING ANAESTHESIA – APPLICATION IN QUALITY IMPROVEMENT AND SAFETY
229. Solfrid Romundstad: EPIDEMIOLOGICAL STUDIES OF MICROALBUMINURIA. THE NORD-TRØNDELAG HEALTH STUDY 1995-97 (HUNT 2)
230. Geir Torheim: PROCESSING OF DYNAMIC DATA SETS IN MAGNETIC RESONANCE IMAGING
231. Catrine Ahlén: SKIN INFECTIONS IN OCCUPATIONAL SATURATION DIVERS IN THE NORTH SEA AND THE IMPACT OF THE ENVIRONMENT
232. Arnulf Langhammer: RESPIRATORY SYMPTOMS, LUNG FUNCTION AND BONE MINERAL DENSITY IN A COMPREHENSIVE POPULATION SURVEY. THE NORD-TRØNDELAG HEALTH STUDY 1995-97. THE BRONCHIAL OBSTRUCTION IN NORD-TRØNDELAG STUDY
233. Einar Kjelsås: EATING DISORDERS AND PHYSICAL ACTIVITY IN NON-CLINICAL SAMPLES
234. Arne Wibe: RECTAL CANCER TREATMENT IN NORWAY – STANDARDISATION OF SURGERY AND QUALITY ASSURANCE

2004

235. Eivind Witsø: BONE GRAFT AS AN ANTIBIOTIC CARRIER
236. Anne Mari Sund: DEVELOPMENT OF DEPRESSIVE SYMPTOMS IN EARLY ADOLESCENCE
237. Hallvard Lærum: EVALUATION OF ELECTRONIC MEDICAL RECORDS – A CLINICAL TASK PERSPECTIVE
238. Gustav Mikkelsen: ACCESSIBILITY OF INFORMATION IN ELECTRONIC PATIENT RECORDS; AN EVALUATION OF THE ROLE OF DATA QUALITY
239. Steinar Krokstad: SOCIOECONOMIC INEQUALITIES IN HEALTH AND DISABILITY. SOCIAL EPIDEMIOLOGY IN THE NORD-TRØNDELAG HEALTH STUDY (HUNT), NORWAY
240. Arne Kristian Myhre: NORMAL VARIATION IN ANOGENITAL ANATOMY AND MICROBIOLOGY IN NON-ABUSED PRESCHOOL CHILDREN
241. Ingunn Dybedal: NEGATIVE REGULATORS OF HEMATOPOIETEC STEM AND PROGENITOR CELLS
242. Beate Sitter: TISSUE CHARACTERIZATION BY HIGH RESOLUTION MAGIC ANGLE SPINNING MR SPECTROSCOPY
243. Per Arne Aas: MACROMOLECULAR MAINTENANCE IN HUMAN CELLS – REPAIR OF URACIL IN DNA AND METHYLATIONS IN DNA AND RNA

244. Anna Bofin: FINE NEEDLE ASPIRATION CYTOLOGY IN THE PRIMARY INVESTIGATION OF BREAST TUMOURS AND IN THE DETERMINATION OF TREATMENT STRATEGIES
245. Jim Aage Nøttestad: DEINSTITUTIONALIZATION AND MENTAL HEALTH CHANGES AMONG PEOPLE WITH MENTAL RETARDATION
246. Reidar Fossmark: GASTRIC CANCER IN JAPANESE COTTON RATS
247. Wibeke Nordhøy: MANGANESE AND THE HEART, INTRACELLULAR MR RELAXATION AND WATER EXCHANGE ACROSS THE CARDIAC CELL MEMBRANE

2005

248. Sturla Molden: QUANTITATIVE ANALYSES OF SINGLE UNITS RECORDED FROM THE HIPPOCAMPUS AND ENTORHINAL CORTEX OF BEHAVING RATS
249. Wenche Brenne Drøyvold: EPIDEMIOLOGICAL STUDIES ON WEIGHT CHANGE AND HEALTH IN A LARGE POPULATION. THE NORD-TRØNDELAG HEALTH STUDY (HUNT)
250. Ragnhild Støen: ENDOTHELIUM-DEPENDENT VASODILATION IN THE FEMORAL ARTERY OF DEVELOPING PIGLETS
251. Aslak Steinsbekk: HOMEOPATHY IN THE PREVENTION OF UPPER RESPIRATORY TRACT INFECTIONS IN CHILDREN
252. Hill-Aina Steffenach: MEMORY IN HIPPOCAMPAL AND CORTICO-HIPPOCAMPAL CIRCUITS
253. Eystein Stordal: ASPECTS OF THE EPIDEMIOLOGY OF DEPRESSIONS BASED ON SELF-RATING IN A LARGE GENERAL HEALTH STUDY (THE HUNT-2 STUDY)
254. Viggo Pettersen: FROM MUSCLES TO SINGING: THE ACTIVITY OF ACCESSORY BREATHING MUSCLES AND THORAX MOVEMENT IN CLASSICAL SINGING
255. Marianne Fyhn: SPATIAL MAPS IN THE HIPPOCAMPUS AND ENTORHINAL CORTEX
256. Robert Valderhaug: OBSESSIVE-COMPULSIVE DISORDER AMONG CHILDREN AND ADOLESCENTS: CHARACTERISTICS AND PSYCHOLOGICAL MANAGEMENT OF PATIENTS IN OUTPATIENT PSYCHIATRIC CLINICS
257. Erik Skaaheim Haug: INFRARENAL ABDOMINAL AORTIC ANEURYSMS – COMORBIDITY AND RESULTS FOLLOWING OPEN SURGERY
258. Daniel Kondziella: GLIAL-NEURONAL INTERACTIONS IN EXPERIMENTAL BRAIN DISORDERS
259. Vegard Heimly Brun: ROUTES TO SPATIAL MEMORY IN HIPPOCAMPAL PLACE CELLS
260. Kenneth McMillan: PHYSIOLOGICAL ASSESSMENT AND TRAINING OF ENDURANCE AND STRENGTH IN PROFESSIONAL YOUTH SOCCER PLAYERS
261. Marit Sæbø Indredavik: MENTAL HEALTH AND CEREBRAL MAGNETIC RESONANCE IMAGING IN ADOLESCENTS WITH LOW BIRTH WEIGHT
262. Ole Johan Kemi: ON THE CELLULAR BASIS OF AEROBIC FITNESS, INTENSITY-DEPENDENCE AND TIME-COURSE OF CARDIOMYOCYTE AND ENDOTHELIAL ADAPTATIONS TO EXERCISE TRAINING
263. Eszter Vanky: POLYCYSTIC OVARY SYNDROME – METFORMIN TREATMENT IN PREGNANCY
264. Hild Fjærtoft: EXTENDED STROKE UNIT SERVICE AND EARLY SUPPORTED DISCHARGE. SHORT AND LONG-TERM EFFECTS
265. Grete Dyb: POSTTRAUMATIC STRESS REACTIONS IN CHILDREN AND ADOLESCENTS
266. Vidar Fykse: SOMATOSTATIN AND THE STOMACH
267. Kirsti Berg: OXIDATIVE STRESS AND THE ISCHEMIC HEART: A STUDY IN PATIENTS UNDERGOING CORONARY REVASCULARIZATION
268. Björn Inge Gustafsson: THE SEROTONIN PRODUCING ENTEROCHROMAFFIN CELL, AND EFFECTS OF HYPERSEROTONINEMIA ON HEART AND BONE

2006

269. Torstein Baade Rø: EFFECTS OF BONE MORPHOGENETIC PROTEINS, HEPATOCYTE GROWTH FACTOR AND INTERLEUKIN-21 IN MULTIPLE MYELOMA
270. May-Britt Tessem: METABOLIC EFFECTS OF ULTRAVIOLET RADIATION ON THE ANTERIOR PART OF THE EYE
271. Anne-Sofie Helvik: COPING AND EVERYDAY LIFE IN A POPULATION OF ADULTS WITH HEARING IMPAIRMENT

272. Therese Standal: MULTIPLE MYELOMA: THE INTERPLAY BETWEEN MALIGNANT PLASMA CELLS AND THE BONE MARROW MICROENVIRONMENT
273. Ingvild Saltvedt: TREATMENT OF ACUTELY SICK, FRAIL ELDERLY PATIENTS IN A GERIATRIC EVALUATION AND MANAGEMENT UNIT – RESULTS FROM A PROSPECTIVE RANDOMISED TRIAL
274. Birger Henning Endreseth: STRATEGIES IN RECTAL CANCER TREATMENT – FOCUS ON EARLY RECTAL CANCER AND THE INFLUENCE OF AGE ON PROGNOSIS
275. Anne Mari Aukan Rokstad: ALGINATE CAPSULES AS BIOREACTORS FOR CELL THERAPY
276. Mansour Akbari: HUMAN BASE EXCISION REPAIR FOR PRESERVATION OF GENOMIC STABILITY
277. Stein Sundstrøm: IMPROVING TREATMENT IN PATIENTS WITH LUNG CANCER – RESULTS FROM TWO MULITCENTRE RANDOMISED STUDIES
278. Hilde Pleym: BLEEDING AFTER CORONARY ARTERY BYPASS SURGERY - STUDIES ON HEMOSTATIC MECHANISMS, PROPHYLACTIC DRUG TREATMENT AND EFFECTS OF AUTOTRANSFUSION
279. Line Merethe Oldervoll: PHYSICAL ACTIVITY AND EXERCISE INTERVENTIONS IN CANCER PATIENTS
280. Boye Welde: THE SIGNIFICANCE OF ENDURANCE TRAINING, RESISTANCE TRAINING AND MOTIVATIONAL STYLES IN ATHLETIC PERFORMANCE AMONG ELITE JUNIOR CROSS-COUNTRY SKIERS
281. Per Olav Vandvik: IRRITABLE BOWEL SYNDROME IN NORWAY, STUDIES OF PREVALENCE, DIAGNOSIS AND CHARACTERISTICS IN GENERAL PRACTICE AND IN THE POPULATION
282. Idar Kirkeby-Garstad: CLINICAL PHYSIOLOGY OF EARLY MOBILIZATION AFTER CARDIAC SURGERY
283. Linn Getz: SUSTAINABLE AND RESPONSIBLE PREVENTIVE MEDICINE. CONCEPTUALISING ETHICAL DILEMMAS ARISING FROM CLINICAL IMPLEMENTATION OF ADVANCING MEDICAL TECHNOLOGY
284. Eva Tegnander: DETECTION OF CONGENITAL HEART DEFECTS IN A NON-SELECTED POPULATION OF 42,381 FETUSES
285. Kristin Gabestad Nørsett: GENE EXPRESSION STUDIES IN GASTROINTESTINAL PATHOPHYSIOLOGY AND NEOPLASIA
286. Per Magnus Haram: GENETIC VS. AQUIRED FITNESS: METABOLIC, VASCULAR AND CARDIOMYOCYTE ADAPTATIONS
287. Agneta Johansson: GENERAL RISK FACTORS FOR GAMBLING PROBLEMS AND THE PREVALENCE OF PATHOLOGICAL GAMBLING IN NORWAY
288. Svein Artur Jensen: THE PREVALENCE OF SYMPTOMATIC ARTERIAL DISEASE OF THE LOWER LIMB
289. Charlotte Björk Ingul: QUANITIFICATION OF REGIONAL MYOCARDIAL FUNCTION BY STRAIN RATE AND STRAIN FOR EVALUATION OF CORONARY ARTERY DISEASE. AUTOMATED VERSUS MANUAL ANALYSIS DURING ACUTE MYOCARDIAL INFARCTION AND DOBUTAMINE STRESS ECHOCARDIOGRAPHY
290. Jakob Nakling: RESULTS AND CONSEQUENCES OF ROUTINE ULTRASOUND SCREENING IN PREGNANCY – A GEOGRAPHIC BASED POPULATION STUDY
291. Anne Engum: DEPRESSION AND ANXIETY – THEIR RELATIONS TO THYROID DYSFUNCTION AND DIABETES IN A LARGE EPIDEMIOLOGICAL STUDY
292. Ottar Bjerkeset: ANXIETY AND DEPRESSION IN THE GENERAL POPULATION: RISK FACTORS, INTERVENTION AND OUTCOME – THE NORD-TRØNDELAG HEALTH STUDY (HUNT)
293. Jon Olav Drogset: RESULTS AFTER SURGICAL TREATMENT OF ANTERIOR CRUCIATE LIGAMENT INJURIES – A CLINICAL STUDY
294. Lars Fosse: MECHANICAL BEHAVIOUR OF COMPACTED MORSELLISED BONE – AN EXPERIMENTAL IN VITRO STUDY
295. Gunilla Klensmeden Fosse: MENTAL HEALTH OF PSYCHIATRIC OUTPATIENTS BULLIED IN CHILDHOOD
296. Paul Jarle Mork: MUSCLE ACTIVITY IN WORK AND LEISURE AND ITS ASSOCIATION TO MUSCULOSKELETAL PAIN

297. Björn Stenström: LESSONS FROM RODENTS: I: MECHANISMS OF OBESITY SURGERY – ROLE OF STOMACH. II: CARCINOGENIC EFFECTS OF *HELICOBACTER PYLORI* AND SNUS IN THE STOMACH

2007

298. Haakon R. Skogseth: INVASIVE PROPERTIES OF CANCER – A TREATMENT TARGET ? IN VITRO STUDIES IN HUMAN PROSTATE CANCER CELL LINES
299. Janniche Hammer: GLUTAMATE METABOLISM AND CYCLING IN MESIAL TEMPORAL LOBE EPILEPSY
300. May Britt Drugli: YOUNG CHILDREN TREATED BECAUSE OF ODD/CD: CONDUCT PROBLEMS AND SOCIAL COMPETENCIES IN DAY-CARE AND SCHOOL SETTINGS
301. Arne Skjold: MAGNETIC RESONANCE KINETICS OF MANGANESE DIPYRIDOXYL DIPHOSPHATE (MnDPDP) IN HUMAN MYOCARDIUM. STUDIES IN HEALTHY VOLUNTEERS AND IN PATIENTS WITH RECENT MYOCARDIAL INFARCTION
302. Siri Malm: LEFT VENTRICULAR SYSTOLIC FUNCTION AND MYOCARDIAL PERFUSION ASSESSED BY CONTRAST ECHOCARDIOGRAPHY
303. Valentina Maria do Rosario Cabral Iversen: MENTAL HEALTH AND PSYCHOLOGICAL ADAPTATION OF CLINICAL AND NON-CLINICAL MIGRANT GROUPS
304. Lasse Løvstakken: SIGNAL PROCESSING IN DIAGNOSTIC ULTRASOUND: ALGORITHMS FOR REAL-TIME ESTIMATION AND VISUALIZATION OF BLOOD FLOW VELOCITY
305. Elisabeth Olstad: GLUTAMATE AND GABA: MAJOR PLAYERS IN NEURONAL METABOLISM
306. Lilian Leistad: THE ROLE OF CYTOKINES AND PHOSPHOLIPASE A_2S IN ARTICULAR CARTILAGE CHONDROCYTES IN RHEUMATOID ARTHRITIS AND OSTEOARTHRITIS
307. Arne Vaaler: EFFECTS OF PSYCHIATRIC INTENSIVE CARE UNIT IN AN ACUTE PSYCIATHRIC WARD
308. Mathias Toft: GENETIC STUDIES OF LRRK2 AND PINK1 IN PARKINSON'S DISEASE
309. Ingrid Løvold Mostad: IMPACT OF DIETARY FAT QUANTITY AND QUALITY IN TYPE 2 DIABETES WITH EMPHASIS ON MARINE N-3 FATTY ACIDS
310. Torill Eidhammer Sjøbakk: MR DETERMINED BRAIN METABOLIC PATTERN IN PATIENTS WITH BRAIN METASTASES AND ADOLESCENTS WITH LOW BIRTH WEIGHT
311. Vidar Beisvåg: PHYSIOLOGICAL GENOMICS OF HEART FAILURE: FROM TECHNOLOGY TO PHYSIOLOGY
312. Olav Magnus Søndenå Fredheim: HEALTH RELATED QUALITY OF LIFE ASSESSMENT AND ASPECTS OF THE CLINICAL PHARMACOLOGY OF METHADONE IN PATIENTS WITH CHRONIC NON-MALIGNANT PAIN
313. Anne Brantberg: FETAL AND PERINATAL IMPLICATIONS OF ANOMALIES IN THE GASTROINTESTINAL TRACT AND THE ABDOMINAL WALL
314. Erik Solligård: GUT LUMINAL MICRODIALYSIS
315. Elin Tollefsen: RESPIRATORY SYMPTOMS IN A COMPREHENSIVE POPULATION BASED STUDY AMONG ADOLESCENTS 13-19 YEARS. YOUNG-HUNT 1995-97 AND 2000-01; THE NORD-TRØNDELAG HEALTH STUDIES (HUNT)
316. Anne-Tove Brenne: GROWTH REGULATION OF MYELOMA CELLS
317. Heidi Knobel: FATIGUE IN CANCER TREATMENT – ASSESSMENT, COURSE AND ETIOLOGY
318. Torbjørn Dahl: CAROTID ARTERY STENOSIS. DIAGNOSTIC AND THERAPEUTIC ASPECTS
319. Inge-Andre Rasmussen jr.: FUNCTIONAL AND DIFFUSION TENSOR MAGNETIC RESONANCE IMAGING IN NEUROSURGICAL PATIENTS
320. Grete Helen Bratberg: PUBERTAL TIMING – ANTECEDENT TO RISK OR RESILIENCE ? EPIDEMIOLOGICAL STUDIES ON GROWTH, MATURATION AND HEALTH RISK BEHAVIOURS; THE YOUNG HUNT STUDY, NORD-TRØNDELAG, NORWAY
321. Sveinung Sørhaug: THE PULMONARY NEUROENDOCRINE SYSTEM. PHYSIOLOGICAL, PATHOLOGICAL AND TUMOURIGENIC ASPECTS
322. Olav Sande Eftedal: ULTRASONIC DETECTION OF DECOMPRESSION INDUCED VASCULAR MICROBUBBLES
323. Rune Bang Leistad: PAIN, AUTONOMIC ACTIVATION AND MUSCULAR ACTIVITY RELATED TO EXPERIMENTALLY-INDUCED COGNITIVE STRESS IN HEADACHE PATIENTS

324. Svein Brekke: TECHNIQUES FOR ENHANCEMENT OF TEMPORAL RESOLUTION IN THREE-DIMENSIONAL ECHOCARDIOGRAPHY
325. Kristian Bernhard Nilsen: AUTONOMIC ACTIVATION AND MUSCLE ACTIVITY IN RELATION TO MUSCULOSKELETAL PAIN
326. Anne Irene Hagen: HEREDITARY BREAST CANCER IN NORWAY. DETECTION AND PROGNOSIS OF BREAST CANCER IN FAMILIES WITH *BRCA1*GENE MUTATION
327. Ingebjørg S. Juel : INTESTINAL INJURY AND RECOVERY AFTER ISCHEMIA. AN EXPERIMENTAL STUDY ON RESTITUTION OF THE SURFACE EPITHELIUM, INTESTINAL PERMEABILITY, AND RELEASE OF BIOMARKERS FROM THE MUCOSA
328. Runa Heimstad: POST-TERM PREGNANCY
329. Jan Egil Afset: ROLE OF ENTEROPATHOGENIC *ESCHERICHIA COLI* IN CHILDHOOD DIARRHOEA IN NORWAY
330. Bent Håvard Hellum: *IN VITRO* INTERACTIONS BETWEEN MEDICINAL DRUGS AND HERBS ON CYTOCHROME P-450 METABOLISM AND P-GLYCOPROTEIN TRANSPORT
331. Morten André Høydal: CARDIAC DYSFUNCTION AND MAXIMAL OXYGEN UPTAKE MYOCARDIAL ADAPTATION TO ENDURANCE TRAINING

2008

332. Andreas Møllerløkken: REDUCTION OF VASCULAR BUBBLES: METHODS TO PREVENT THE ADVERSE EFFECTS OF DECOMPRESSION
333. Anne Hege Aamodt: COMORBIDITY OF HEADACHE AND MIGRAINE IN THE NORD-TRØNDELAG HEALTH STUDY 1995-97
334. Brage Høyem Amundsen: MYOCARDIAL FUNCTION QUANTIFIED BY SPECKLE TRACKING AND TISSUE DOPPLER ECHOCARDIOGRAPHY – VALIDATION AND APPLICATION IN EXERCISE TESTING AND TRAINING
335. Inger Anne Næss: INCIDENCE, MORTALITY AND RISK FACTORS OF FIRST VENOUS THROMBOSIS IN A GENERAL POPULATION. RESULTS FROM THE SECOND NORD-TRØNDELAG HEALTH STUDY (HUNT2)
336. Vegard Bugten: EFFECTS OF POSTOPERATIVE MEASURES AFTER FUNCTIONAL ENDOSCOPIC SINUS SURGERY
337. Morten Bruvold: MANGANESE AND WATER IN CARDIAC MAGNETIC RESONANCE IMAGING
338. Miroslav Fris: THE EFFECT OF SINGLE AND REPEATED ULTRAVIOLET RADIATION ON THE ANTERIOR SEGMENT OF THE RABBIT EYE
339. Svein Arne Aase: METHODS FOR IMPROVING QUALITY AND EFFICIENCY IN QUANTITATIVE ECHOCARDIOGRAPHY – ASPECTS OF USING HIGH FRAME RATE
340. Roger Almvik: ASSESSING THE RISK OF VIOLENCE: DEVELOPMENT AND VALIDATION OF THE BRØSET VIOLENCE CHECKLIST
341. Ottar Sundheim: STRUCTURE-FUNCTION ANALYSIS OF HUMAN ENZYMES INITIATING NUCLEOBASE REPAIR IN DNA AND RNA
342. Anne Mari Undheim: SHORT AND LONG-TERM OUTCOME OF EMOTIONAL AND BEHAVIOURAL PROBLEMS IN YOUNG ADOLESCENTS WITH AND WITHOUT READING DIFFICULTIES
343. Helge Garåsen: THE TRONDHEIM MODEL. IMPROVING THE PROFESSIONAL COMMUNICATION BETWEEN THE VARIOUS LEVELS OF HEALTH CARE SERVICES AND IMPLEMENTATION OF INTERMEDIATE CARE AT A COMMUNITY HOSPITAL COULD PROVIDE BETTER CARE FOR OLDER PATIENTS. SHORT AND LONG TERM EFFECTS
344. Olav A. Foss: "THE ROTATION RATIOS METHOD". A METHOD TO DESCRIBE ALTERED SPATIAL ORIENTATION IN SEQUENTIAL RADIOGRAPHS FROM ONE PELVIS
345. Bjørn Olav Åsvold: THYROID FUNCTION AND CARDIOVASCULAR HEALTH
346. Torun Margareta Melø: NEURONAL GLIAL INTERACTIONS IN EPILEPSY
347. Irina Poliakova Eide: FETAL GROWTH RESTRICTION AND PRE-ECLAMPSIA: SOME CHARACTERISTICS OF FETO-MATERNAL INTERACTIONS IN DECIDUA BASALIS
348. Torunn Askim: RECOVERY AFTER STROKE. ASSESSMENT AND TREATMENT; WITH FOCUS ON MOTOR FUNCTION
349. Ann Elisabeth Åsberg: NEUTROPHIL ACTIVATION IN A ROLLER PUMP MODEL OF CARDIOPULMONARY BYPASS. INFLUENCE ON BIOMATERIAL, PLATELETS AND COMPLEMENT

350. Lars Hagen: REGULATION OF DNA BASE EXCISION REPAIR BY PROTEIN INTERACTIONS AND POST TRANSLATIONAL MODIFICATIONS
351. Sigrun Beate Kjøtrød: POLYCYSTIC OVARY SYNDROME – METFORMIN TREATMENT IN ASSISTED REPRODUCTION
352. Steven Keita Nishiyama: PERSPECTIVES ON LIMB-VASCULAR HETEROGENEITY: IMPLICATIONS FOR HUMAN AGING, SEX, AND EXERCISE
353. Sven Peter Näsholm: ULTRASOUND BEAMS FOR ENHANCED IMAGE QUALITY
354. Jon Ståle Ritland: PRIMARY OPEN-ANGLE GLAUCOMA & EXFOLIATIVE GLAUCOMA. SURVIVAL, COMORBIDITY AND GENETICS
355. Sigrid Botne Sando: ALZHEIMER'S DISEASE IN CENTRAL NORWAY. GENETIC AND EDUCATIONAL ASPECTS
356. Parvinder Kaur: CELLULAR AND MOLECULAR MECHANISMS BEHIND METHYLMERCURY-INDUCED NEUROTOXICITY
357. Ismail Cüneyt Güzey: DOPAMINE AND SEROTONIN RECEPTOR AND TRANSPORTER GENE POLYMORPHISMS AND EXTRAPYRAMIDAL SYMPTOMS. STUDIES IN PARKINSON'S DISEASE AND IN PATIENTS TREATED WITH ANTIPSYCHOTIC OR ANTIDEPRESSANT DRUGS
358. Brit Dybdahl: EXTRA-CELLULAR INDUCIBLE HEAT-SHOCK PROTEIN 70 (Hsp70) – A ROLE IN THE INFLAMMATORY RESPONSE ?
359. Kristoffer Haugarvoll: IDENTIFYING GENETIC CAUSES OF PARKINSON'S DISEASE IN NORWAY
360. Nadra Nilsen: TOLL-LIKE RECEPTOR 2 –EXPRESSION, REGULATION AND SIGNALING
361. Johan Håkon Bjørngaard: PATIENT SATISFACTION WITH OUTPATIENT MENTAL HEALTH SERVICES – THE INFLUENCE OF ORGANIZATIONAL FACTORS.
362. Kjetil Høydal : EFFECTS OF HIGH INTENSITY AEROBIC TRAINING IN HEALTHY SUBJECTS AND CORONARY ARTERY DISEASE PATIENTS; THE IMPORTANCE OF INTENSITY,, DURATION AND FREQUENCY OF TRAINING.
363. Trine Karlsen: TRAINING IS MEDICINE: ENDURANCE AND STRENGTH TRAINING IN CORONARY ARTERY DISEASE AND HEALTH.
364. Marte Thuen: MANGANASE-ENHANCED AND DIFFUSION TENSOR MR IMAGING OF THE NORMAL, INJURED AND REGENERATING RAT VISUAL PATHWAY
365. Cathrine Broberg Vågbø: DIRECT REPAIR OF ALKYLATION DAMAGE IN DNA AND RNA BY 2-OXOGLUTARATE- AND IRON-DEPENDENT DIOXYGENASES
366. Arnt Erik Tjønna: AEROBIC EXERCISE AND CARDIOVASCULAR RISK FACTORS IN OVERWEIGHT AND OBESE ADOLESCENTS AND ADULTS
367. Marianne W. Furnes: FEEDING BEHAVIOR AND BODY WEIGHT DEVELOPMENT: LESSONS FROM RATS
368. Lene N. Johannessen: FUNGAL PRODUCTS AND INFLAMMATORY RESPONSES IN HUMAN MONOCYTES AND EPITHELIAL CELLS
369. Anja Bye: GENE EXPRESSION PROFILING OF *INHERITED* AND *ACQUIRED* MAXIMAL OXYGEN UPTAKE – RELATIONS TO THE METABOLIC SYNDROME.
370. Oluf Dimitri Røe: MALIGNANT MESOTHELIOMA: VIRUS, BIOMARKERS AND GENES. A TRANSLATIONAL APPROACH
371. Ane Cecilie Dale: DIABETES MELLITUS AND FATAL ISCHEMIC HEART DISEASE. ANALYSES FROM THE HUNT1 AND 2 STUDIES
372. Jacob Christian Hølen: PAIN ASSESSMENT IN PALLIATIVE CARE: VALIDATION OF METHODS FOR SELF-REPORT AND BEHAVIOURAL ASSESSMENT
373. Erming Tian: THE GENETIC IMPACTS IN THE ONCOGENESIS OF MULTIPLE MYELOMA
374. Ole Bosnes: KLINISK UTPRØVING AV NORSKE VERSJONER AV NOEN SENTRALE TESTER PÅ KOGNITIV FUNKSJON
375. Ola M. Rygh: 3D ULTRASOUND BASED NEURONAVIGATION IN NEUROSURGERY. A CLINICAL EVALUATION
376. Astrid Kamilla Stunes: ADIPOKINES, PEROXISOME PROFILERATOR ACTIVATED RECEPTOR (PPAR) AGONISTS AND SEROTONIN. COMMON REGULATORS OF BONE AND FAT METABOLISM
377. Silje Engdal: HERBAL REMEDIES USED BY NORWEGIAN CANCER PATIENTS AND THEIR ROLE IN HERB-DRUG INTERACTIONS
378. Kristin Offerdal: IMPROVED ULTRASOUND IMAGING OF THE FETUS AND ITS CONSEQUENCES FOR SEVERE AND LESS SEVERE ANOMALIES

379. Øivind Rognmo: HIGH-INTENSITY AEROBIC EXERCISE AND CARDIOVASCULAR HEALTH
380. Jo-Åsmund Lund: RADIOTHERAPY IN ANAL CARCINOMA AND PROSTATE CANCER

2009

381. Tore Grüner Bjåstad: HIGH FRAME RATE ULTRASOUND IMAGING USING PARALLEL BEAMFORMING
382. Erik Søndenaa: INTELLECTUAL DISABILITIES IN THE CRIMINAL JUSTICE SYSTEM
383. Berit Rostad: SOCIAL INEQUALITIES IN WOMEN'S HEALTH, HUNT 1984-86 AND 1995-97, THE NORD-TRØNDELAG HEALTH STUDY (HUNT)
384. Jonas Crosby: ULTRASOUND-BASED QUANTIFICATION OF MYOCARDIAL DEFORMATION AND ROTATION
385. Erling Tronvik: MIGRAINE, BLOOD PRESSURE AND THE RENIN-ANGIOTENSIN SYSTEM
386. Tom Christensen: BRINGING THE GP TO THE FOREFRONT OF EPR DEVELOPMENT
387. Håkon Bergseng: ASPECTS OF GROUP B STREPTOCOCCUS (GBS) DISEASE IN THE NEWBORN. EPIDEMIOLOGY, CHARACTERISATION OF INVASIVE STRAINS AND EVALUATION OF INTRAPARTUM SCREENING
388. Ronny Myhre: GENETIC STUDIES OF CANDIDATE TENE3S IN PARKINSON'S DISEASE
389. Torbjørn Moe Eggebø: ULTRASOUND AND LABOUR
390. Eivind Wang: TRAINING IS MEDICINE FOR PATIENTS WITH PERIPHERAL ARTERIAL DISEASE
391. Thea Kristin Våtsveen: GENETIC ABERRATIONS IN MYELOMA CELLS
392. Thomas Jozefiak: QUALITY OF LIFE AND MENTAL HEALTH IN CHILDREN AND ADOLESCENTS: CHILD AND PARENT PERSPECTIVES
393. Jens Erik Slagsvold: N-3 POLYUNSATURATED FATTY ACIDS IN HEALTH AND DISEASE – CLINICAL AND MOLECULAR ASPECTS
394. Kristine Misund: A STUDY OF THE TRANSCRIPTIONAL REPRESSOR ICER. REGULATORY NETWORKS IN GASTRIN-INDUCED GENE EXPRESSION
395. Franco M. Impellizzeri: HIGH-INTENSITY TRAINING IN FOOTBALL PLAYERS. EFFECTS ON PHYSICAL AND TECHNICAL PERFORMANCE
396. Kari Hanne Gjeilo: HEALTH-RELATED QUALITY OF LIFE AND CHRONIC PAIN IN PATIENTS UNDERGOING CARDIAC SURGERY
397. Øyvind Hauso: NEUROENDOCRINE ASPECTS OF PHYSIOLOGY AND DISEASE
398. Ingvild Bjellmo Johnsen: INTRACELLULAR SIGNALING MECHANISMS IN THE INNATE IMMUNE RESPONSE TO VIRAL INFECTIONS
399. Linda Tømmerdal Roten: GENETIC PREDISPOSITION FOR DEVELOPMENT OF PREEMCLAMPSIA – CANDIDATE GENE STUDIES IN THE HUNT (NORD-TRØNDELAG HEALTH STUDY) POPULATION
400. Trude Teoline Nausthaug Rakvåg: PHARMACOGENETICS OF MORPHINE IN CANCER PAIN
401. Hanne Lehn: MEMORY FUNCTIONS OF THE HUMAN MEDIAL TEMPORAL LOBE STUDIED WITH fMRI
402. Randi Utne Holt: ADHESION AND MIGRATION OF MYELOMA CELLS – IN VITRO STUDIES –
403. Trygve Solstad: NEURAL REPRESENTATIONS OF EUCLIDEAN SPACE
404. Unn-Merete Fagerli: MULTIPLE MYELOMA CELLS AND CYTOKINES FROM THE BONE MARROW ENVIRONMENT; ASPECTS OF GROWTH REGULATION AND MIGRATION
405. Sigrid Bjørnelv: EATING– AND WEIGHT PROBLEMS IN ADOLESCENTS, THE YOUNG HUNT-STUDY
406. Mari Hoff: CORTICAL HAND BONE LOSS IN RHEUMATOID ARTHRITIS. EVALUATING DIGITAL X-RAY RADIOGRAMMETRY AS OUTCOME MEASURE OF DISEASE ACTIVITY, RESPONSE VARIABLE TO TREATMENT AND PREDICTOR OF BONE DAMAGE
407. Siri Bjørgen: AEROBIC HIGH INTENSITY INTERVAL TRAINING IS AN EFFECTIVE TREATMENT FOR PATIENTS WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASE
408. Susanne Lindqvist: VISION AND BRAIN IN ADOLESCENTS WITH LOW BIRTH WEIGHT
409. Torbjørn Hergum: 3D ULTRASOUND FOR QUANTITATIVE ECHOCARDIOGRAPHY

410. Jørgen Urnes: PATIENT EDUCATION IN GASTRO-OESOPHAGEAL REFLUX DISEASE. VALIDATION OF A DIGESTIVE SYMPTOMS AND IMPACT QUESTIONNAIRE AND A RANDOMISED CONTROLLED TRIAL OF PATIENT EDUCATION
411. Elvar Eyjolfsson: 13C NMRS OF ANIMAL MODELS OF SCHIZOPHRENIA
412. Marius Steiro Fimland: CHRONIC AND ACUTE NEURAL ADAPTATIONS TO STRENGTH TRAINING
413. Øyvind Støren: RUNNING AND CYCLING ECONOMY IN ATHLETES; DETERMINING FACTORS, TRAINING INTERVENTIONS AND TESTING
414. Håkon Hov: HEPATOCYTE GROWTH FACTOR AND ITS RECEPTOR C-MET. AUTOCRINE GROWTH AND SIGNALING IN MULTIPLE MYELOMA CELLS
415. Maria Radtke: ROLE OF AUTOIMMUNITY AND OVERSTIMULATION FOR BETA-CELL DEFICIENCY. EPIDEMIOLOGICAL AND THERAPEUTIC PERSPECTIVES
416. Liv Bente Romundstad: ASSISTED FERTILIZATION IN NORWAY: SAFETY OF THE REPRODUCTIVE TECHNOLOGY
417. Erik Magnus Berntsen: PREOPERATIV PLANNING AND FUNCTIONAL NEURONAVIGATION – WITH FUNCTIONAL MRI AND DIFFUSION TENSOR TRACTOGRAPHY IN PATIENTS WITH BRAIN LESIONS
418. Tonje Strømmen Steigedal: MOLECULAR MECHANISMS OF THE PROLIFERATIVE RESPONSE TO THE HORMONE GASTRIN
419. Vidar Rao: EXTRACORPOREAL PHOTOCHEMOTHERAPY IN PATIENTS WITH CUTANEOUS T CELL LYMPHOMA OR GRAFT-vs-HOST DISEASE
420. Torkild Visnes: DNA EXCISION REPAIR OF URACIL AND 5-FLUOROURACIL IN HUMAN CANCER CELL LINES

2010

421. John Munkhaugen: BLOOD PRESSURE, BODY WEIGHT, AND KIDNEY FUNCTION IN THE NEAR-NORMAL RANGE: NORMALITY, RISK FACTOR OR MORBIDITY ?
422. Ingrid Castberg: PHARMACOKINETICS, DRUG INTERACTIONS AND ADHERENCE TO TREATMENT WITH ANTIPSYCHOTICS: STUDIES IN A NATURALISTIC SETTING
423. Jian Xu: BLOOD-OXYGEN-LEVEL-DEPENDENT-FUNCTIONAL MAGNETIC RESONANCE IMAGING AND DIFFUSION TENSOR IMAGING IN TRAUMATIC BRAIN INJURY RESEARCH
424. Sigmund Simonsen: ACCEPTABLE RISK AND THE REQUIREMENT OF PROPORTIONALITY IN EUROPEAN BIOMEDICAL RESEARCH LAW. WHAT DOES THE REQUIREMENT THAT BIOMEDICAL RESEARCH SHALL NOT INVOLVE RISKS AND BURDENS DISPROPORTIONATE TO ITS POTENTIAL BENEFITS MEAN?
425. Astrid Woodhouse: MOTOR CONTROL IN WHIPLASH AND CHRONIC NON-TRAUMATIC NECK PAIN
426. Line Rørstad Jensen: EVALUATION OF TREATMENT EFFECTS IN CANCER BY MR IMAGING AND SPECTROSCOPY
427. Trine Moholdt: AEROBIC EXERCISE IN CORONARY HEART DISEASE
428. Øystein Olsen: ANALYSIS OF MANGANESE ENHANCED MRI OF THE NORMAL AND INJURED RAT CENTRAL NERVOUS SYSTEM
429. Bjørn H. Grønberg: PEMETREXED IN THE TREATMENT OF ADVANCED LUNG CANCER
430. Vigdis Schnell Husby: REHABILITATION OF PATIENTS UNDERGOING TOTAL HIP ARTHROPLASTY WITH FOCUS ON MUSCLE STRENGTH, WALKING AND AEROBIC ENDURANCE PERFORMANCE
431. Torbjørn Øien: CHALLENGES IN PRIMARY PREVENTION OF ALLERGY. THE PREVENTION OF ALLERGY AMONG CHILDREN IN TRONDHEIM (PACT) STUDY.
432. Kari Anne Indredavik Evensen: BORN TOO SOON OR TOO SMALL: MOTOR PROBLEMS IN ADOLESCENCE
433. Lars Adde: PREDICTION OF CEREBRAL PALSY IN YOUNG INFANTS. COMPUTER BASED ASSESSMENT OF GENERAL MOVEMENTS
434. Magnus Fasting: PRE- AND POSTNATAL RISK FACTORS FOR CHILDHOOD ADIPOSITY
435. Vivi Talstad Monsen: MECHANISMS OF ALKYLATION DAMAGE REPAIR BY HUMAN AlkB HOMOLOGUES
436. Toril Skandsen: MODERATE AND SEVERE TRAUMATIC BRAIN INJURY. MAGNETIC RESONANCE IMAGING FINDINGS, COGNITION AND RISK FACTORS FOR DISABILITY

437. Ingeborg Smidesang: ALLERGY RELATED DISORDERS AMONG 2-YEAR OLDS AND ADOLESCENTS IN MID-NORWAY – PREVALENCE, SEVERITY AND IMPACT. THE PACT STUDY 2005, THE YOUNG HUNT STUDY 1995-97
438. Vidar Halsteinli: MEASURING EFFICIENCY IN MENTAL HEALTH SERVICE DELIVERY: A STUDY OF OUTPATIENT UNITS IN NORWAY
439. Karen Lehrmann Ægidius: THE PREVALENCE OF HEADACHE AND MIGRAINE IN RELATION TO SEX HORMONE STATUS IN WOMEN. THE HUNT 2 STUDY
440. Madelene Ericsson: EXERCISE TRAINING IN GENETIC MODELS OF HEART FAILURE
441. Marianne Klokk: THE ASSOCIATION BETWEEN SELF-REPORTED ECZEMA AND COMMON MENTAL DISORDERS IN THE GENERAL POPULATION. THE HORDALAND HEALTH STUDY (HUSK)
442. Tomas Ottemo Stølen: IMPAIRED CALCIUM HANDLING IN ANIMAL AND HUMAN CARDIOMYOCYTES REDUCE CONTRACTILITY AND INCREASE ARRHYTHMIA POTENTIAL – EFFECTS OF AEROBIC EXERCISE TRAINING
443. Bjarne Hansen: ENHANCING TREATMENT OUTCOME IN COGNITIVE BEHAVIOURAL THERAPY FOR OBSESSIVE COMPULSIVE DISORDER: THE IMPORTANCE OF COGNITIVE FACTORS
444. Mona Løvlien: WHEN EVERY MINUTE COUNTS. FROM SYMPTOMS TO ADMISSION FOR ACUTE MYOCARDIAL INFARCTION WITH SPECIAL EMPHASIS ON GENDER DIFFERECES
445. Karin Margaretha Gilljam: DNA REPAIR PROTEIN COMPLEXES, FUNCTIONALITY AND SIGNIFICANCE FOR REPAIR EFFICIENCY AND CELL SURVIVAL
446. Anne Byriel Walls: NEURONAL GLIAL INTERACTIONS IN CEREBRAL ENERGY – AND AMINO ACID HOMEOSTASIS – IMPLICATIONS OF GLUTAMATE AND GABA
447. Cathrine Fallang Knetter: MECHANISMS OF TOLL-LIKE RECEPTOR 9 ACTIVATION
448. Marit Følsvik Svindseth: A STUDY OF HUMILIATION, NARCISSISM AND TREATMENT OUTCOME IN PATIENTS ADMITTED TO PSYCHIATRIC EMERGENCY UNITS
449. Karin Elvenes Bakkelund: GASTRIC NEUROENDOCRINE CELLS – ROLE IN GASTRIC NEOPLASIA IN MAN AND RODENTS
450. Kirsten Brun Kjelstrup: DORSOVENTRAL DIFFERENCES IN THE SPATIAL REPRESENTATION AREAS OF THE RAT BRAIN
451. Roar Johansen: MR EVALUATION OF BREAST CANCER PATIENTS WITH POOR PROGNOSIS
452. Rigmor Myran: POST TRAUMATIC NECK PAIN. EPIDEMIOLOGICAL, NEURORADIOLOGICAL AND CLINICAL ASPECTS
453. Krisztina Kunszt Johansen: GENEALOGICAL, CLINICAL AND BIOCHEMICAL STUDIES IN *LRRK2* – ASSOCIATED PARKINSON'S DISEASE
454. Pål Gjerden: THE USE OF ANTICHOLINERGIC ANTIPARKINSON AGENTS IN NORWAY. EPIDEMIOLOGY, TOXICOLOGY AND CLINICAL IMPLICATIONS
455. Else Marie Huuse: ASSESSMENT OF TUMOR MICROENVIRONMENT AND TREATMENT EFFECTS IN HUMAN BREAST CANCER XENOGRAFTS USING MR IMAGING AND SPECTROSCOPY
456. Khalid S. Ibrahim: INTRAOPERATIVE ULTRASOUND ASSESSMENT IN CORONARY ARTERY BYPASS SURGERY – WITH SPECIAL REFERENCE TO CORONARY ANASTOMOSES AND THE ASCENDING AORTA
457. Bjørn Øglænd: ANTHROPOMETRY, BLOOD PRESSURE AND REPRODUCTIVE DEVELOPMENT IN ADOLESCENCE OF OFFSPRING OF MOTHERS WHO HAD PREECLAMPSIA IN PREGNANCY
458. John Olav Roaldset: RISK ASSESSMENT OF VIOLENT, SUICIDAL AND SELF-INJURIOUS BEHAVIOUR IN ACUTE PSYCHIATRY – A BIO-PSYCHO-SOCIAL APPROACH
459. Håvard Dalen: ECHOCARDIOGRAPHIC INDICES OF CARDIAC FUNCTION – NORMAL VALUES AND ASSOCIATIONS WITH CARDIAC RISK FACTORS IN A POPULATION FREE FROM CARDIOVASCULAR DISEASE, HYPERTENSION AND DIABETES: THE HUNT 3 STUDY
460. Beate André: CHANGE CAN BE CHALLENGING. INTRODUCTION TO CHANGES AND IMPLEMENTATION OF COMPUTERIZED TECHNOLOGY IN HEALTH CARE
461. Latha Nrugham: ASSOCIATES AND PREDICTORS OF ATTEMPTED SUICIDE AMONG DEPRESSED ADOLESCENTS – A 6-YEAR PROSPECTIVE STUDY

462. Håvard Bersås Nordgaard: TRANSIT-TIME FLOWMETRY AND WALL SHEAR STRESS ANALYSIS OF CORONARY ARTERY BYPASS GRAFTS – A CLINICAL AND EXPERIMENTAL STUDY

Cotutelle with University of Ghent: Abigail Emily Swillens: A MULTIPHYSICS MODEL FOR IMPROVING THE ULTRASONIC ASSESSMENT OF LARGE ARTERIES

2011

463. Marte Helene Bjørk: DO BRAIN RHYTHMS CHANGE BEFORE THE MIGRAINE ATTACK? A LONGITUDINAL CONTROLLED EEG STUDY
464. Carl-Jørgen Arum: A STUDY OF UROTHELIAL CARCINOMA: GENE EXPRESSION PROFILING, TUMORIGENESIS AND THERAPIES IN ORTHOTOPIC ANIMAL MODELS
465. Ingunn Harstad: TUBERCULOSIS INFECTION AND DISEASE AMONG ASYLUM SEEKERS IN NORWAY. SCREENING AND FOLLOW-UP IN PUBLIC HEALTH CARE
466. Leif Åge Strand: EPIDEMIOLOGICAL STUDIES AMONG ROYAL NORWEGIAN NAVY SERVICEMEN. COHORT ESTABLISHMENT, CANCER INCIDENCE AND CAUSE-SPECIFIC MORTALITY
467. Katrine Høyer Holgersen: SURVIVORS IN THEIR THIRD DECADE AFTER THE NORTH SEA OIL RIG DISASTER OF 1980. LONG-TERM PERSPECTIVES ON MENTAL HEALTH
468. MarianneWallenius: PREGNANCY RELATED ASPECTS OF CHRONIC INFLAMMATORY ARTHRITIDES: DISEASE ONSET POSTPARTUM, PREGNANCY OUTCOMES AND FERTILITY. DATA FROM A NORWEGIAN PATIENT REGISTRY LINKED TO THE MEDICAL BIRTH REGISTRY OF NORWAY
469. Ole Vegard Solberg: 3D ULTRASOUND AND NAVIGATION – APPLICATIONS IN LAPAROSCOPIC SURGERY
470. Inga Ekeberg Schjerve: EXERCISE-INDUCED IMPROVEMENT OF MAXIMAL OXYGEN UPTAKE AND ENDOTHELIAL FUNCTION IN OBESE AND OVERWEIGHT INDIVIDUALS ARE DEPENDENT ON EXERCISE-INTENSITY
471. Eva Veslemøy Tyldum: CARDIOVASCULAR FUNCTION IN PREECLAMPSIA – WITH REFERENCE TO ENDOTHELIAL FUNCTION, LEFT VENTRICULAR FUNCTION AND PRE-PREGNANCY PHYSICAL ACTIVITY
472. Benjamin Garzón Jiménez de Cisneros: CLINICAL APPLICATIONS OF MULTIMODAL MAGNETIC RESONANCE IMAGING
473. Halvard Knut Nilsen: ASSESSING CODEINE TREATMENT TO PATIENTS WITH CHRONIC NON-MALIGNANT PAIN: NEUROPSYCHOLOGICAL FUNCTIONING, DRIVING ABILITY AND WEANING
474. Eiliv Brenner: GLUTAMATE RELATED METABOLISM IN ANIMAL MODELS OF SCHIZOPHRENIA
475. Egil Jonsbu: CHEST PAIN AND PALPITATIONS IN A CARDIAC SETTING; PSYCHOLOGICAL FACTORS, OUTCOME AND TREATMENT
476. Mona Høysæter Fenstad: GENETIC SUSCEPTIBILITY TO PREECLAMPSIA : STUDIES ON THE NORD-TRØNDELAG HEALTH STUDY (HUNT) COHORT, AN AUSTRALIAN/NEW ZEALAND FAMILY COHORT AND DECIDUA BASALIS TISSUE
477. Svein Erik Gaustad: CARDIOVASCULAR CHANGES IN DIVING: FROM HUMAN RESPONSE TO CELL FUNCTION
478. Karin Torvik: PAIN AND QUALITY OF LIFE IN PATIENTS LIVING IN NURSING HOMES
479. Arne Solberg: OUTCOME ASSESSMENTS IN NON-METASTATIC PROSTATE CANCER
480. Henrik Sahlin Pettersen: CYTOTOXICITY AND REPAIR OF URACIL AND 5-FLUOROURACIL IN DNA
481. Pui-Lam Wong: PHYSICAL AND PHYSIOLOGICAL CAPACITY OF SOCCER PLAYERS: EFFECTS OF STRENGTH AND CONDITIONING
482. Ole Solheim: ULTRASOUND GUIDED SURGERY IN PATIENTS WITH INTRACRANIAL TUMOURS
483. Sten Roar Snare: QUANTITATIVE CARDIAC ANALYSIS ALGORITHMS FOR POCKET-SIZED ULTRASOUND DEVICES
484. Marit Skyrud Bratlie: LARGE-SCALE ANALYSIS OF ORTHOLOGS AND PARALOGS IN VIRUSES AND PROKARYOTES
485. Anne Elisabeth F. Isern: BREAST RECONSTRUCTION AFTER MASTECTOMY – RISK OF RECURRENCE AFTER DELAYED LARGE FLAP RECONSTRUCTION – AESTHETIC OUTCOME, PATIENT SATISFACTION, QUALITY OF LIFE AND SURGICAL RESULTS;

HISTOPATHOLOGICAL FINDINGS AND FOLLOW-UP AFTER PROPHYLACTIC MASTECTOMY IN HEREDITARY BREAST CANCER

486. Guro L. Andersen: CEREBRAL PALSY IN NORWAY – SUBTYPES, SEVERITY AND RISK FACTORS
487. Frode Kolstad: CERVICAL DISC DISEASE – BIOMECHANICAL ASPECTS
488. Bente Nordtug: CARING BURDEN OF COHABITANTS LIVING WITH PARTNERS SUFFERING FROM CHRONIC OBSTRUCTIVE PULMONARY DISEASE OR DEMENTIA
489. Mariann Gjervik Heldahl: EVALUATION OF NEOADJUVANT CHEMOTHERAPY IN LOCALLY ADVANCED BREAST CANCER BASED ON MR METHODOLOGY
490. Lise Tevik Løvseth: THE SUBJECTIVE BURDEN OF CONFIDENTIALITY
491. Marie Hjelmseth Aune: INFLAMMATORY RESPONSES AGAINST GRAM NEGATIVE BACTERIA INDUCED BY TLR4 AND NLRP12
492. Tina Strømdal Wik: EXPERIMENTAL EVALUATION OF NEW CONCEPTS IN HIP ARTHROPLASTY
493. Solveig Sigurdardottir: CLINICAL ASPECTS OF CEREBRAL PALSY IN ICELAND. A POPULATION-BASED STUDY OF PRESCHOOL CHILDREN
494. Arne Reimers: CLINICAL PHARMACOKINETICS OF LAMOTRIGINE
495. Monica Wegling: KULTURMENNESKETS BYRDE OG SYKDOMMENS VELSIGNELSE. KAN MEDISINSK UTREDNING OG INTERVENSJON HA EN SELVSTENDIG FUNKSJON UAVHENGIG AV DET KURATIVE?
496. Silje Alvestad: ASTROCYTE-NEURON INTERACTIONS IN EXPERIMENTAL MESIAL TEMPORAL LOBE EPILEPSY – A STUDY OF UNDERLYING MECHANISMS AND POSSIBLE BIOMARKERS OF EPILEPTOGENESIS
497. Javaid Nauman: RESTING HEART RATE: A MATTER OF LIFE OR DEATH – PROSPECTIVE STUDIES OF RESTING HEART RATE AND CARDIOVASCULAR RISK (THE HUNT STUDY, NORWAY)
498. Thuy Nguyen: THE ROLE OF C-SRC TYROSINE KINASE IN ANTIVIRAL IMMUNE RESPONSES
499. Trine Naalsund Andreassen: PHARMACOKINETIC, PHARMACODYNAMIC AND PHARMACOGENETIC ASPECTS OF OXYCODONE TREATMENT IN CANCER PAIN
500. Eivor Alette Laugsand: SYMPTOMS IN PATIENTS RECEIVING OPIOIDS FOR CANCER PAIN – CLINICAL AND PHARMACOGENETIC ASPECTS
501. Dorthe Stensvold: PHYSICAL ACTIVITY, CARDIOVASCULAR HEALTH AND LONGEVITY IN PATIENTS WITH METABOLIC SYNDROME
502. Stian Thoresen Aspenes: PEAK OXYGEN UPTAKE AMONG HEALTHY ADULTS – CROSS-SECTIONAL DESCRIPTIONS AND PROSPECTIVE ANALYSES OF PEAK OXYGEN UPTAKE, PHYSICAL ACTIVITY AND CARDIOVASCULAR RISK FACTORS IN HEALTHY ADULTS (20-90 YEARS)
503. Reidar Alexander Vigen: PATHOBIOLOGY OF GASTRIC CARCINOIDS AND ADENOCARCINOMAS IN RODENT MODELS AND PATIENTS. STUDIES OF GASTROCYSTOPLASTY, GENDER-RELATED FACTORS, AND AUTOPHAGY
504. Halvard Høilund-Kaupang: MODELS AND METHODS FOR INVESTIGATION OF REVERBERATIONS IN NONLINEAR ULTRASOUND IMAGING
505. Audhild Løhre: WELLBEING AMONG SCHOOL CHILDREN IN GRADES 1-10: PROMOTING AND ADVERSE FACTORS
506. Torgrim Tandstad: VOX POPULI. POPULATION-BASED OUTCOME STUDIES IN TESTICULAR CANCER
507. Anna Brenne Grønskag: THE EPIDEMIOLOGY OF HIP FRACTURES AMONG ELDERLY WOMEN IN NORD-TRØNDELAG. HUNT 1995-97, THE NORD-TRØNDELAG HEALTH STUDY
508. Kari Ravndal Risnes: BIRTH SIZE AND ADULT MORTALITY: A SYSTEMATIC REVIEW AND A LONG-TERM FOLLOW-UP OF NEARLY 40 000 INDIVIDUALS BORN AT ST. OLAV UNIVERSITY HOSPITAL IN TRONDHEIM 1920-1960
509. Hans Jakob Bøe: LONG-TERM POSTTRAUMATIC STRESS AFTER DISASTER – A CONTROLLED STUDY OF SURVIVORS' HEALTH 27 YEARS AFTER THE CAPSIZED NORTH SEA OIL RIG
510. Cathrin Barbara Canto, Cotutelle with University of Amsterdam: LAYER SPECIFIC INTEGRATIVE PROPERTIES OF ENTORHINAL PRINCIPAL NEURONS
511. Ioanna Sandvig: THE ROLE OF OLFACTORY ENSHEATHING CELLS, MRI, AND BIOMATERIALS IN TRANSPLANT-MEDIATED CNS REPAIR

512. Karin Fahl Wader: HEPATOCYTE GROWTH FACTOR, C-MET AND SYNDECAN-1 IN MULTIPLE MYELOMA
513. Gerd Tranø: FAMILIAL COLORECTAL CANCER
514. Bjarte Bergstrøm: INNATE ANTIVIRAL IMMUNITY – MECHANISMS OF THE RIG-I-MEDIATED RESPONSE
515. Marie Søfteland Sandvei: INCIDENCE, MORTALITY, AND RISK FACTORS FOR ANEURYSMAL SUBARACHNOID HEMORRHAGE. PROSPECTIVE ANALYZES OF THE HUNT AND TROMSØ STUDIES
516. Mary-Elizabeth Bradley Eilertsen: CHILDREN AND ADOLESCENTS SURVIVING CANCER: PSYCHOSOCIAL HEALTH, QUALITY OF LIFE AND SOCIAL SUPPORT
517. Takaya Saito: COMPUTATIONAL ANALYSIS OF REGULATORY MECHANISM AND INTERACTIONS OF MICRORNAS

Godkjent for disputas, publisert post mortem: Eivind Jullumstrø: COLORECTAL CANCER AT LEVANGER HOSPITAL 1980-2004

518. Christian Gutvik: A PHYSIOLOGICAL APPROACH TO A NEW DECOMPRESSION ALGORITHM USING NONLINEAR MODEL PREDICTIVE CONTROL
519. Ola Storrø: MODIFICATION OF ADJUVANT RISK FACTOR BEHAVIOURS FOR ALLERGIC DISEASE AND ASSOCIATION BETWEEN EARLY GUT MICROBIOTA AND ATOPIC SENSITIZATION AND ECZEMA. EARLY LIFE EVENTS DEFINING THE FUTURE HEALTH OF OUR CHILDREN
520. Guro Fanneløb Giskeødegård: IDENTIFICATION AND CHARACTERIZATION OF PROGNOSTIC FACTORS IN BREAST CANCER USING MR METABOLOMICS
521. Gro Christine Christensen Løhaugen: BORN PRETERM WITH VERY LOW BIRTH WEIGHT – NEVER ENDING COGNITIVE CONSEQUENCES?
522. Sigrid Nakrem: MEASURING QUALITY OF CARE IN NURSING HOMES – WHAT MATTERS?
523. Brita Pukstad: CHARACTERIZATION OF INNATE INFLAMMATORY RESPONSES IN ACUTE AND CHRONIC WOUNDS

2012

524. Hans H. Wasmuth: ILEAL POUCHES
525. Inger Økland: BIASES IN SECOND-TRIMESTER ULTRASOUND DATING RELATED TO PREDICTION MODELS AND FETAL MEASUREMENTS
526. Bjørn Mørkedal: BLOOD PRESSURE, OBESITY, SERUM IRON AND LIPIDS AS RISK FACTORS OF ISCHAEMIC HEART DISEASE
527. Siver Andreas Moestue: MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF BREAST CANCER THROUGH A COMBINATION OF MR IMAGING, TRANSCRIPTOMICS AND METABOLOMICS
528. Guro Aune: CLINICAL, PATHOLOGICAL, AND MOLECULAR CLASSIFICATION OF OVARIAN CARCINOMA
529. Ingrid Alsos Lian: MECHANISMS INVOLVED IN THE PATHOGENESIS OF PRE-ECLAMPSIA AND FETAL GROWTH RESTRICTION. TRANSCRIPTIONAL ANALYSES OF PLACENTAL AND DECIDUAL TISSUE
530. Karin Solvang-Garten: X-RAY REPAIR CROSS-COMPLEMENTING PROTEIN 1 – THE ROLE AS A SCAFFOLD PROTEIN IN BASE EXCISION REPAIR AND SINGLE STRAND BREAK REPAIR
531. Toril Holien: BONE MORPHOGENETIC PROTEINS AND MYC IN MULTIPLE MYELOMA
532. Rooyen Mavenyengwa: *STREPTOCOCCUS AGALACTIAE* IN PREGNANT WOMEN IN ZIMBABWE: EPIDEMIOLOGY AND SEROTYPE MARKER CHARACTERISTICS
533. Tormod Rimehaug: EMOTIONAL DISTRESS AND PARENTING AMONG COMMUNITY AND CLINIC PARENTS
534. Maria Dung Cao: MR METABOLIC CHARACTERIZATION OF LOCALLY ADVANCED BREAST CANCER – TREATMENT EFFECTS AND PROGNOSIS
535. Mirta Mittelstedt Leal de Sousa: PROTEOMICS ANALYSIS OF PROTEINS INVOLVED IN DNA BASE REPAIR AND CANCER THERAPY
536. Halfdan Petursson: THE VALIDITY AND RELEVANCE OF INTERNATIONAL CARDIOVASCULAR DISEASE PREVENTION GUIDELINES FOR GENERAL PRACTICE
537. Marit By Rise: LIFTING THE VEIL FROM USER PARTICIPATION IN CLINICAL WORK – WHAT IS IT AND DOES IT WORK?

538. Lene Thoresen: NUTRITION CARE IN CANCER PATIENTS. NUTRITION ASSESSMENT: DIAGNOSTIC CRITERIA AND THE ASSOCIATION TO SURVIVAL AND HEALTH-RELATED QUALITY OF LIFE IN PATIENTS WITH ADVANCED COLORECTAL CARCINOMA
539. Berit Doseth: PROCESSING OF GENOMIC URACIL IN MAN AND MOUSE
540. Gro Falkenér Bertheussen: PHYSICAL ACTIVITY AND HEALTH IN A GENERAL POPULATION AND IN CANCER SURVIVORS – METHODOLOGICAL, OBSERVATIONAL AND CLINICAL ASPECTS
541. Anne Kari Knudsen: CANCER PAIN CLASSIFICATION
542. Sjur Urdson Gjerald: A FAST ULTRASOUND SIMULATOR
543. Harald Edvard Mølmen Hansen: CARDIOVASCULAR EFFECTS OF HIGH INTENSITY AEROBIC INTERVAL TRAINING IN HYPERTENSITIVE PATIENTS, HEALTHY AGED AND YOUNG PERSONS
544. Sasha Gulati: SURGICAL RESECTION OF HIGH-GRADE GLIOMAS
545. John Chr. Fløvig: FREQUENCY AND EFFECT OF SUBSTANCES AND PSYCHOACTIVE MEDICATIONS THE WEEK BEFORE ADMISSION TO AN ACUTE PSYCHIATRIC DEPARTMENT
546. Kristin Moksnes Husby: OPTIMIZING OPIOID TREATMENT FOR CANCER PAIN – CLINICAL AND PHARMACOLOGICAL ASPECTS
547. Audun Hanssen-Bauer: X-RAY REPAIR CROSS-COMPLEMENTING PROTEIN 1 ASSOCIATED MULTIPROTEIN COMPLEXES IN BASE EXCISION REPAIR
548. Marit Saunes: ECZEMA IN CHILDREN AND ADOLESCENTS – EPIDEMIOLOGY, COURSE AND IMPACT. THE PREVENTION OF ALLERGY AMONG CHILDREN IN TRONDHEIM (PACT) STUDY, YOUNG-HUNT 1995-97
549. Guri Kaurstad: CARDIOMYOCYTE FUNCTION AND CALCIUM HANDLING IN ANIMAL MODELS OF INBORN AND ACQUIRED MAXIMAL OXYGEN UPTAKE
550. Kristian Svendsen: METHODOLOGICAL CHALLENGES IN PHARMACOEPIDEMIOLOGICAL STUDIES OF OPIOID CONSUMPTION
551. Signe Nilssen Stafne: EXERCISE DURING PREGNANCY
552. Marius Widerøe: MAGNETIC RESONANCE IMAGING OF HYPOXIC-ISCHEMIC BRAIN INJURY DEVELOPMENT IN THE NEWBORN RAT – MANGANESE AND DIFFUSION CONTRASTS
553. Andreas Radtke: MOLECULAR METHODS FOR TYPING *STREPTOCOCCUS AGALACTIAE* WITH SPECIAL EMPHASIS ON THE DEVELOPMENT AND VALIDATION OF A MULTI-LOCUS VARIABLE NUMBER OF TANDEM REPEATS ASSAY (MLVA)