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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,