Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Paraclinical Sciences

Philosophiae Doctor (PhD)
Thesis 2022:63

Piecing together the chain of infection for *Streptococcus dysgalactiae* in sheep and dairy cows - risk factors, sources, and genomic diversity of bacterial strains

Undersøkelser av infeksjonskjeden for *Streptococcus dysgalactiae*-infeksjoner i norsk sauehold og melkekubesetninger - risikofaktorer, kilder og genomisk diversitet

Marit Smistad

Piecing together the chain of infection for *Streptococcus dysgalactiae* in sheep and dairy cows

- risk factors, sources, and genomic diversity of bacterial strains

Undersøkelser av infeksjonskjeden for *Streptococcus dysgalactiae*-infeksjoner i norsk sauehold og melkekubesetninger
- risikofaktorer, kilder og genomisk diversitet

Philosophiae Doctor (PhD) Thesis

Marit Smistad

Norwegian University of Life Sciences

Faculty of Veterinary Medicine

Department of Paraclinical Sciences

Ås (2022)

Thesis number 2022:63

ISSN 1894-6402

ISBN 978-82-575-2015-1

Supervisors and Evaluation Committee

Supervisors

Hannah Joan Jørgensen
Dr.med.vet, PhD
Section for Animal Health, Welfare and Food Safety
Norwegian Veterinary Institute

Trine L'Abée-Lund
Associate professor
Department of Paraclinical Sciences
Norwegian University of Life Sciences

Cecilia M. Wolff
Dr.med.vet, PhD
Section for Animal Health, Welfare and Food Safety
Norwegian Veterinary Institute

Davide Porcellato, NMBU
Associate professor
Faculty of Chemistry, Biotechnology and Food Science
Norwegian University of Life Sciences

Anne Cathrine Whist, TINE
Dr.med.vet, PhD
TINE SA

Evaluation committee

Ylva Persson
State Veterinarian, National Veterinary Institute/
Associate Professor, Swedish University of Agricultural Sciences

Jennifer Duncan
Senior lecturer
University of Liverpool

Hans Petter Kjæstad
Associate professor
Norwegian University of Life Sciences

Acknowledgements

This work was part of an industrial Ph.D. scheme carried out between 2019 and 2022, where I was employed in TINE SA, conducted the work at the Norwegian Veterinary Institute, and was enrolled in the Ph.D. program of the Department of Paraclinical Sciences, Faculty of Veterinary Medicine at the Norwegian University of Life Sciences. The PhD project was part of the research project "Increasing sustainability of Norwegian food production by tackling streptococcal infections in modern livestock systems", coordinated by the Norwegian Veterinary Institute. It received financial contributions from the Norwegian Agricultural Agreement Research Fund and the Norwegian Research council (grant numbers 280364 and 288917), Animalia, and TINE SA. I am grateful that I was given the opportunity to do this PhD and for the facilities and funding provided for me to do this work.

Thank you, Hannah Joan Jørgensen, for your solid guidance and support all the way from start to completion of this work. Your endless knowledge in the bacteriology field, combined with your enthusiasm and fantastic sense of humour, have been a true inspiration. Thank you for always having answers to my questions, ranging from bacterial population structures to how to raise children. Thank you for all the time you have invested in me.

Thank you, Liv Sølverød, for being the wisest mentor and the most inspiring leader I could have, for all the nerdy discussions on udder health and diagnostics, and for always responding "yes", whatever I ask.

Cecilia Wolff – thank you for everything you have taught me about epidemiology (and tables), for always being positive and for giving me some encouraging words when I needed them the most.

Thank you, Basma Asal and Fiona Valerie Franklin-Alming, for all the hours you have spent at the lab looking for "de små blå" ("the small blue ones"). Thank you for sharing your passion for bacteriology and for the patience in teaching me basic laboratory skills.

Thank you to the staff at TINE mastitis laboratory for all you have taught me about mastitis diagnostics, all the milk samples you have collected in the project, and for always making me feel welcome in Molde.

A special thanks to Ravithas Kathirgamadas and the staff at the section for Substrate Production and Logistics at the Norwegian Veterinary Institute for excellent service and for always deliver the strangest media, including autoclaved slurry.

Thank you, Lars Austbø and colleagues at the Department of Molecular Biology at the Norwegian Veterinary Institute for developing the qPCR and being invaluable partners in the molecular part of the work.

Thank you to the two most intelligent people I know, Davide Porcellato and Håkon Kaspersen – for patiently attempting to introduce the simple dairy veterinary advisor to the world of genomics.

Tore Tollersrud and Vibeke Tømmerberg in Animalia are acknowledged for contributing with their enormous knowledge and enthusiasm for sheep and for all the work done before the PhD-project, giving me a flying start.

Trine L'Abée-Lund, Anne Cathrine Whist, and all co-authors of the included papers are greatly acknowledged for their contributions. The many farmers and veterinarians willingly contributing to our research are greatly acknowledged.

Olav Østerås – thank you for sharing your many ideas, inspiring me with your magic with data, and always believing in me and supporting me. Tormod Mørk – thank you for bringing all your wise ideas into the project and being available as a one-of-a-kind sparring partner with knowledge spanning from the barn to the genomes during the period.

Thank you, colleagues at section 122 at the Veterinary Institute and the research department of TINE, for providing an inspiring work environment.

A big thanks to my friends and family for your constant support these four years. Thank you, Torunn and Finn, for all the help with our kids and dogs. Without you, finishing on time would not be possible. Thank you, Herman, Iver, and Luna – for forcing me to take many (!) breaks and reminding me what really matters. To my dad, thanks for letting me grow up around animals and inspiring me to become

a veterinarian. Finn Petter, thank you for standing by me in these extremely busy years. I could not have done this without you.

Ås, August 2022

Marit Smistad

Table of Contents

1 Abbreviations and definitions

AMS: Automatic milking system

AMR: Antimicrobial resistance

IMI: Intramammary infection

MLST: Multi locus sequence typing

NHRS: Norwegian herd recording system

NGS: Next generation sequencing

NSRS: Norwegian Sheep Recording system

NVI: Norwegian Veterinary Institute

PFGE: Pulsed-field gel electrophoresis

SD: *Streptococcus dysgalactiae*

SCC: Somatic cell count

SDSD: *Streptococcus dysgalactiae* subspecies *dysgalactiae*

SDSE: *Streptococcus dysgalactiae* subspecies *equisimilis*

SNPs: Single nucleotide polymorphisms

ST: Sequence Type

WGS: Whole genome sequencing

2 List of papers

I. Smistad, M., Wolff, C., Tollersrud, T., Tømmerberg, V., Phythian, C., Kampen, A. H., & Jørgensen, H. J. (2020). Flock-level risk factors for outbreaks of infectious arthritis in lambs, Norway 2018. Acta Veterinaria Scandinavica, 62(1), 1-11.

II. Smistad, M., Tollersrud, T. S., Austbø, L., Porcellato, D., Wolff, C., Asal, B., Phythian, C. J., Oppegaard, O., & Jørgensen, H. J. (2021). Molecular detection and genotype characterization of *Streptococcus dysgalactiae* from sheep flocks with outbreaks of infectious arthritis. Veterinary Microbiology, 262, 109221.

III. Smistad, M., Kaspersen, H., Franklin-Alming, F. V., Wolff, C., Sølverød, L., Porcellato, D., Trettenes, E., & Jørgensen, H. J. (2022). *Streptococcus dysgalactiae* ssp. *dysgalactiae* in Norwegian bovine dairy herds: Risk factors, sources, and genomic diversity. Journal of Dairy Science, 105(4), 3574-3587.

IV. Porcellato, D., Smistad, M., Skeie, S. B., Jørgensen, H. J., Austbø, L., & Oppegaard, O. (2021). Whole genome sequencing reveals possible host species adaptation of *Streptococcus dysgalactiae*. Scientific Reports, 11(1), 1-13.

3 Abstract

Streptococcus dysgalactiae subspecies *dysgalactiae* (SDSD) is an important cause of outbreaks of infectious arthritis in lambs and mastitis in dairy cows in Norway. The infections compromise animal welfare, increase antimicrobial usage and reduce production. The Norwegian sheep and dairy industries state that streptococcal infections are one of the major challenges to production.

The aim of this work was to increase our understanding of how SDSD spreads and causes disease in in sheep and cattle in Norway. We approached the aim through risk factor studies, exploration of bacterial sources, and genomic investigations of bacterial isolates from Norwegian sheep flocks and bovine dairy herds.

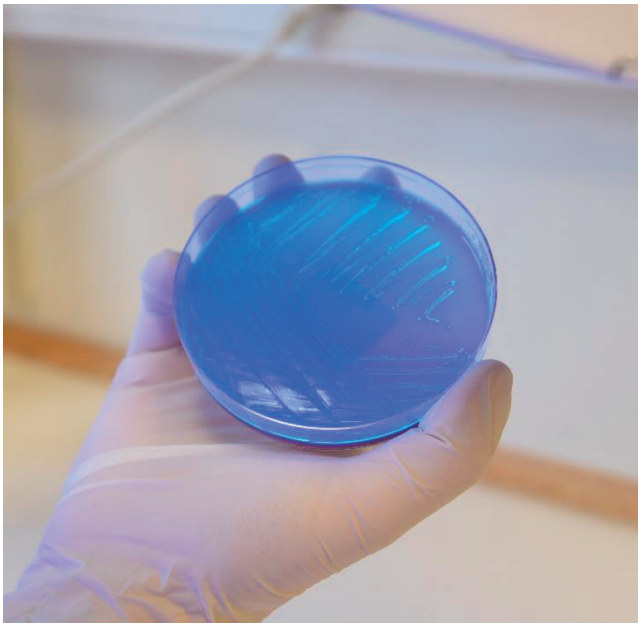
Several risk factors related to modern management systems were identified. Larger sheep flocks with a lambing percentage above 200 had an increased risk of outbreaks of infectious arthritis in lambs. Intramammary infections caused by SDSD were more common in bovine dairy herds housed in freestalls compared to tiestalls. Certain types of flooring were a risk factor for SDSD infections in both sheep flocks (plastic mesh flooring) and bovine dairy herds (closed flooring).

Analyses of samples collected from sheep flocks and bovine dairy herds revealed that SDSD is present in most of the visited sheep flocks and bovine dairy herds. SDSD is mainly associated with the host, but was also found in the environment. A greater proportion of environmental samples from bovine freestalls were SDSD positive compared to samples collected from sheep sheds during lambing. Wounds were particularly often colonized by SDSD in both animal species and may serve as a site of bacterial multiplication that increases the size of the bacterial reservoir. Prevention of wounds may therefore be an important measure to reduce SDSD infection pressure.

Genomic investigations revealed a clonal population structure of the SDSD, and isolates were delineated according to host species. Highly similar strains were found in epidemiologically independent flocks and herds. We found no significant association between genotype and disease severity, defined as clinical mastitis in cows (compared to subclinical mastitis) and outbreaks of infectious arthritis in lambs.

In conclusion, this study indicates that SDSD is an animal-adapted opportunist that has lived with the hosts over time. The work has contributed to our understanding of

risk factors, sources, and transmission dynamics in modern management systems and also the genome and population structure of SDSD. It provides the basis for updated advice in the animal health services and will thus contribute to reducing SDSD-infections in Norwegian livestock, reducing animal suffering, and increasing productivity.



De små blå [the small blue ones]. Photo: Bryndis Holm, Norwegian Veterinary Institute

4 Norsk sammendrag

Streptococcus dysgalactiae subspecies *dysgalactiae* (SDSD) er en viktig årsak til utbrudd av leddbetennelse hos lam og mastitt hos melkekyr i Norge. Infeksjonene fører til redusert dyrevelferd, økt forbruk av antibiotika og reduserer produksjonsutbyttet.

Målet med dette arbeidet var å øke vår forståelse av hvordan SDSD-infeksjoner spres og gir sykdom hos sau og melkeku i Norge. For å oppnå målet gjennomførte vi risikofaktorstudier, undersøkte bakteriekilder og genomiske undersøkelser av bakterieisolater fra norske saueflokker og storfebesetninger.

Flere risikofaktorer knyttet til moderne driftsformer ble identifisert. Større saueflokker med mer enn to lam per søye hadde økt risiko for utbrudd av smittsom leddbetennelse hos lam. Intramammare infeksjoner forårsaket av SDSD var mer vanlig i melkekubesetninger som ble holdt i løsdriftsfjøs sammenlignet med båsfjøs. Visse typer gulv var en risikofaktor for SDSD-infeksjoner i både saueflokker (plastrister) og melkekufjøs (tett gulv).

Analyser av prøver samlet inn fra saueflokker og melkekubesetninger viste at SDSD er til stede i de fleste besøkte besetningene. Bakterien ser ut til å trives best på verten, men synes også å overleve en stund i miljøet. En større andel av miljøprøver fra melkekufjøs var SDSD-positive sammenlignet med prøver samlet inn fra sauefjøs under lamming. Sår ble spesielt ofte kolonisert av SDSD hos begge dyrearter, og kan fungere som et oppformeringssted for bakterien. Forebygging av sår kan derfor være et viktig tiltak for å redusere infeksjonspresset.

Undersøkelser av bakteriens arvestoff viste at SDSD har en klonal populasjonsstruktur, hvor isolatene fra en vertsart var mer like innbyrdes. Svært like varianter ble funnet i epidemiologisk uavhengige flokker og besetninger. Resultatene indikerer at SDSD er en dyretilpasset opportunist som har levd med vertene over tid. Vi fant ingen signifikant sammenheng mellom genotype og sykdomsgrad, definert som klinisk mastitt hos kyr (sammenlignet med subklinisk mastitt) og utbrudd av leddbetennelse hos lam.

Arbeidet gir grunnlag for oppdaterte råd for norske saue- og melkekuprodusenter, og vil bidra til å redusere SDSD-infeksjoner i norske husdyr, bedre dyrevelferd og øke produktiviteten.

5 Introduction

5.1 Background

Norway has approximately 13,400 sheep farms and 7,200 bovine dairy farms spread across the country (Statistics Norway, 2022). With only three percent cultivable land, the sheep and dairy industry play a crucial role in Norwegian agriculture by utilizing non-cultivable land for grazing.

Overall, Norway has good animal health and low antimicrobial consumption. The climate, the geographic location and topography, as well as strict regulations for live animal import are likely to have contributed to this. The political goal of self-sufficiency of meat and milk has encouraged the maintenance of small, geographically widespread farms. However, structural changes in the livestock industry have also occurred in Norway, from demands for increased productivity. With modernization, specialization, and alterations in production and management, new challenges have arisen.

The incidence of streptococcal infections has increased in Norwegian sheep and dairy cows in the past decades. Infections caused by *Streptococcus dysgalactiae* subspecies *dysgalactiae* (SDSD) are a common concern for both sectors, but basic knowledge regarding how this bacterium transmits and causes disease is limited. Updated advice on how to prevent SDSD infections in modern livestock systems is needed.

5.1.1 The Norwegian sheep industry

Measured in the number of farms, the Norwegian sheep industry constitutes the largest sector of Norwegian livestock production, with 950,000 sheep in 13,400 farms. The average flock size is 67 breeding ewes (Statistics Norway, 2022). Sheep are kept for meat and wool, with the composite crossbreed Norwegian White Sheep accounting for 70% of the national population.

Due to the climate, most Norwegian sheep are housed during the winter from mating until after lambing. A minimum grazing period of 16 weeks for sheep is mandatory (Norwegian Ministry of Agriculture and Food, 2005), and outfield grazing presently represents 40% of the total feed (Nortura, 2019).

Agricultural subsidies are a prerequisite for Norway's sheep industry. Farmers receive direct payments depending on the number of breeding ewes. Adjustments of

the system for economic compensations have influenced flock sizes throughout the years.

5.1.2 The Norwegian bovine dairy industry

The Norwegian dairy industry comprises 213,000 dairy cows in approximately 7,200 farms. The average herd size is 29 cows, and the average annual milk yield is 8,204 kg (Statistics Norway, 2022). Norwegian red, a dual-purpose breed, accounts for 92% of the national herd (TINE, 2020).

A quota system regulates national milk production, and the maximum milk production per farm is 400,000 litres. In 2020, 64% of the dairy cows were in freestall housing, and 57% of the milk was produced in automatic milking systems (AMS) (TINE, 2020). Norwegian dairy cows are housed during the winter, and the mandatory pasture season is eight and 16 weeks for freestall and tiestall herds, respectively (Norwegian Ministry of Agriculture and Food, 2004).

Norwegian dairy farmers have a tradition of participating in the Norwegian Herd Recording System (NHRS), which currently includes milk recordings, disease treatments, and meat production from 98% of the national dairy herds (TINE, 2020). The NHRS data have informed breeding and herd improvement programs and have most likely contributed to improved health of the Norwegian dairy cow population.

5.1.3 Modernization of the sheep and dairy industries of Norway

The Norwegian sheep and dairy industry have followed the same trend as the livestock production in other industrialized countries, with a move towards fewer and larger farms. The number of sheep farms has declined from 21,500 in 2000 to 13,400 in 2022, while the number of dairy farms has decreased from more than 20,000 farms in 2000 to approximately 7,000 farms in 2021. The total production volume for both sectors has remained relatively constant due to increased herd sizes and increased production per unit (Statistics Norway, 2022).

Previously, the upper limit of direct payment per sheep was 300 ewes. The authorities removed this limit to stimulate production in 2013. This led to the development of sheep farms that were significantly larger than the traditional farms. Typically, these farms are well managed and have a high production per sheep, frequently with lambing percentages of 250 (Tømmerberg et al., 2017). With several hundreds of lambs born indoors within a few weeks, stocking densities become high, and it is challenging to keep the indoor environment clean and dry during lambing.

The dairy industry is also going through an extensive restructuring, contributed by a regulatory enforced transition from tiestall to freestall housing within 2034 (Norwegian Ministry of Agriculture and Food, 2004). Many farmers invest in larger freestall buildings and AMS, and rely on a higher production per cow to cover the costs.

5.1.4 New challenges in modern sheep and dairy production

Animal health and welfare in modern sheep and dairy production have improved in many ways. Farmers of modern sheep and dairy farms often manage to sustain a full-time job at the farm, and management has become professionalised. Optimisation of feeding and adoption of standard operating procedures have contributed to reduced occurrence of diseases like ketosis and milk fever, which previously constituted a considerable proportion of disease treatments in traditional tiestalls (TINE, 2020). The Norwegian dairy population is also free from important infectious diseases like paratuberculosis and bovine viral diarrhoea. However, other infections have become more problematic in recent years.

Many infectious diseases develop in a complex interplay between the host, pathogen, and environment, often referred to as *the epidemiological triad* (Figure 1). An increasing prevalence of specific infections, like SDSD, may follow from exposure to new risk factors, introduction of new variants of the infectious agent, or more susceptible hosts.

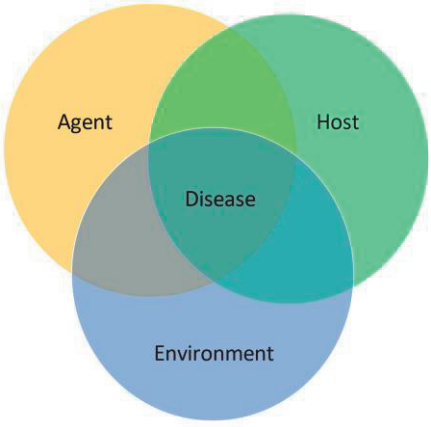


Figure 1: Infectious disease may be understood through the epidemiological triad. Figure adapted from Van Seventer and Hochberg, 2017.

5.2 *Streptococcus dysgalactiae*

Streptococcus dysgalactiae (SD) is a facultative anaerobic, Gram-positive coccus belonging to the group of pyogenic streptococci and is divided into two subspecies: *Streptococcus dysgalactiae* subspecies *dysgalactiae* (SDSD) and subspecies *equisimilis* (SDSE). The name originates from the Greek word streptos and kokkos, which means "chains of cocci", and *dysgalactiae* refers to decreased milk production. The name was first used by Diernhofer in 1932, describing a streptococcus isolated from bovine mastitis (Diernhofer, 1932).

SDSD is mainly associated with infections in animals, including mastitis in dairy cows (Ericsson Unnerstad et al., 2009; Kabelitz et al., 2021; Vakkamäki et al., 2017) and infectious arthritis and septicaemia in lambs (Angus, 1991; Rutherford et al., 2015; Watkins and Sharp, 1998). SDSE, on the other hand, is an emerging pathogen of humans, with a wide spectrum of clinical manifestations, including tonsillitis, soft tissue infections, infectious arthritis, and septicaemia (Oppegaard et al., 2017). SDSE is also associated with disease in horses (Pinho et al., 2016), pigs (Oh et al., 2020), and dogs (Lamm et al., 2010). Streptococci are susceptible to most prescribed antimicrobials, including penicillin (Vos et al., 2011).

SDSD was traditionally identified based on colony morphology and haemolysis pattern, and can be phenotypically distinguished from SDSE as described in Table 1. The colonies are small (approximately 1 mm in diameter) and grey with alpha-haemolysis on blood agar after 24 hours of incubation at 37 °C. The inability to degrade aesculin is a crucial trait differentiating SDSD from *Streptococcus uberis* and *Streptococcus agalactiae*. Lancefield serological grouping, based on the composition of bacterial antigens in the cell wall, can also help differentiate streptococcal species (Table 1).

Table 1: Principles for differentiation of the closely related streptococci Streptococcus dysgalactiae *subspecies* dysgalactiae *(SDSD),* Streptococcus dysgalactiae *subspecies* equisimilis *(SDSE) and* Streptococcus canis (S. canis)

Bacterial species	**Lancefield**	**Haemolysis pattern on blood agar***	**Hosts**
SDSD	C	alpha	Mainly sheep and cattle**
SDSE	A, C, G and L	beta	Humans and warm-blood animals **
S. canis	G	beta	Mainly dogs and cats**

*5% sheep or horse blood agar

**sporadically in other species, including fish (uncertain species confirmation)

In recent years, mass spectrometry for colony identification has revolutionised diagnostics. Matrix-Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) determines the unique proteomic fingerprint of a bacterium and matches it with a reference library to determine the species. However, it cannot identify SD beyond the species level. Therefore, colony morphology, haemolysis pattern and aesculin reaction are still used to distinguish SDSD and SDSE, and the closely related *Streptococcus canis*.

5.2.1 Hosts and reservoirs

The ability of a pathogen to cause disease is determined by an interplay between agent, host, and environmental factors. The spread of an infectious agent in a population requires a sequence of events, often referred to as *the chain of infection* (Figure 2). Preventive measures should focus on breaking one or more links of the chain to stop the spread of disease.

A bacterium can be part of the host's microbiome and cause opportunistic disease under certain conditions or be strict pathogens with the main reservoir in diseased animals or asymptomatic carriers. Some bacteria require a host to survive, whilst others may persist outside the host for extended periods. For strict pathogens, measures to prevent the introduction or achieve eradication from a herd may be relevant. Eradicating or preventing the introduction of opportunistic bacteria belonging to the normal bacterial flora is likely to be unsuccessful.

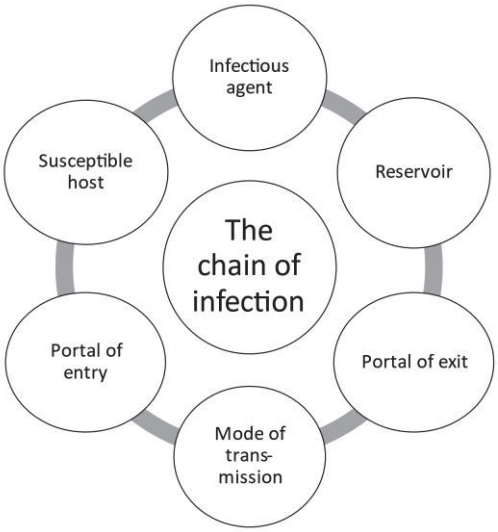


Figure 2: The chain of infection illustrates the events required for transmission of infectious disease. Figure adapted from (Van Seventer and Hochberg, 2017).

Knowledge of a bacterium's natural habitat, and potential reservoirs, is necessary to understand its transmission. The main niches of SDSD are unknown. Most studies have focused on identification of SDSD for diagnostic purposes, either in milk samples as a cause of intramammary infections (IMI) in dairy cows (Duse et al., 2021; Olde Riekerink et al., 2008; Vakkamäki et al., 2017) or in joint aspirates from lambs with infectious arthritis (Watkins and Sharp, 1998; Watt and Refshauge, 2010). In addition, reports describe sporadic cases of arthritis and septicaemia in calves (Ryan et al., 1991), infective endocarditis and prosthetic joint infection in humans (Jordal et al., 2015; Park et al., 2012) and neonatal sepsis in puppies (Vela et al., 2006).

In bovine mastitis, a distinction is often made between contagious and environmental udder pathogens (Zadoks et al., 2011). Contagious udder pathogens have their main reservoir in infected udder quarters and depend on the host to survive and spread. Milk from infected udders represents the portal of exit for the pathogen, and transmission often occurs at milking, including at preparation (Neave et al., 1969). Environmental udder pathogens have their primary reservoir in the environment and are able to multiply there. Transmission occurs between milkings (Zadoks and Schukken, 2006). Some authors consider SDSD a contagious udder pathogen (Barkema et al., 1999; Fuquay et al., 2011; Olde Riekerink et al., 2008), while others classify it as environmental (Smith and Hogan, 1993; Todhunter et al., 1995). The fact

that many new SDSD-IMI are seen in heifers or dry cows (Schwan and Holmberg, 1979; Todhunter et al., 1995; Persson Waller et al., 2009), which are usually not stalled with lactating animals, may indicate that sources other than milk from infected cows play a role in transmission.

The literature on reservoirs of SDSD outside infected udders and joints, such as other body sites or the environment, is sparse. In sheep flocks, the bacterium has been identified occasionally in wounds (Blakemore et al., 1941), navels (Blakemore et al., 1941; Cornell and Glover, 1925), milk from one ewe (Lacasta et al., 2008), the vagina of ewes (Blakemore, 1939; Rutherford et al., 2014), and the skin of lambs (Rutherford et al., 2014). With respect to SDSD transmission in sheep flocks, the portal of exit is less obvious than for IMI in cattle, as most lesions in joints are closed. In bovine dairy herds, SDSD has been detected in tonsils (Cruz Colque et al., 1993; Daleel and Frost, 1967), the skin and wound of one cow (Lundberg, 2015), and flies (Bramley et al., 1985; Chirico et al., 1997).

The identification of colonization sites, reservoirs, and portals of exit for other pyogenic streptococci have contributed to the understanding of pathogenesis and transmission. Vaginal carriage of *S. agalactiae* in humans is an essential part of the pathogenesis of neonatal sepsis, where the infant may acquire the infection in the birth canal (Schuchat, 1999). Studies of soldiers in military barracks indicated that colonization of *S. pyogenens* of dry, intact skin was rare, while nasopharyngeal carriage was a significant source of contamination of the environment (Hamburger et al., 1945; Lemon et al., 1948). However, broken skin, such as wounds or chronic skin conditions predisposes to clinical disease caused by *S. pyogenes*. Hamburger (1947) proposed direct contact by handshaking as a likely transmission route between carriers and non-carriers.

Streptococcus uberis, also a well-known cause of bovine mastitis, is widely distributed in the barn environment (Bramley, 1982; Cullen, 1966). However, based on the difference in contamination rates between high and low cow traffic areas (Lopez-Benavides et al., 2007), and failure to find *S. uberis* in non-farm soil (Zadoks et al., 2005), it is suggested that the presence of cows is needed for the initial inoculum of the environment. It is proposed that faecal shedding plays a role in contamination the environment (Kruze and Bramley, 1982; Lopez-Benavides et al., 2007), and that hygienic measures to decrease contact between udders and manure can prevent *S. uberis* IMI.

For decades, milk from *S. agalactiae*-infected udders was believed to be the only source of the bacterium in dairy herds (Keefe, 2012). However, many farmers failed to eradicate *S. agalactiae* despite targeted treatment and culling of cows with IMI. The discovery of *S. agalactiae* in the faeces, water troughs, and slurry in the barn environment led to updated recommendations for Norwegian dairy farmers, targeting these extramammary sources with hygienic measures (Jørgensen et al., 2016).

The role of a potential environmental reservoir for SDSD is unclear. Previous attempts to detect SDSD in the environment of dairy cows (Lundberg, 2015) and sheep (Rutherford et al., 2014) have been unsuccessful. It has been unknown whether the inability to detect SDSD in environmental samples is due to the low sensitivity of detection methods or that the bacterium does not thrive in the environment. Streptococci have complex nutritional requirements (Vos et al., 2011), a shorter survival time on inanimate surfaces, and are more vulnerable to desiccation compared to staphylococci (Miescher et al., 1955; Wißmann et al., 2021). For SDSD, few survival-studies have been performed. Rutherford (2014) demonstrated that SDSD could survive for at least 42 days in clean, dry straw bedding in vitro but could not recover SDSD in sterile water inoculated with SDSD.

5.2.2 Infectious arthritis in lambs

Arthritis means inflammation in one or more joints. In lambs, it is characterized by joint swelling and lameness and may or may not be coupled with systemic signs like fever or lethargy. Most cases of arthritis in lambs are caused by bacterial infections (Craig et al., 2015). Infectious arthritis may be further classified according to the nature of the exudate and the host response and the duration of the lesion (Craig et al., 2015; Watkins, 2007).

Several opportunistic bacterial species may be involved in infectious arthritis in lambs, among which *Erysipelothrix rhusiopathiae*, *Trueperella pyogenes*, *Escherichia coli,* and *Staphylococcus aureus* are of importance. The particular role of streptococci in outbreaks of infectious arthritis in lambs has been recognized since the early 1900s (Blakemore, 1939; Cornell and Glover, 1925).

Infectious arthritis caused by SDSD is characterized by an acute onset of lameness or recumbency in lambs under four weeks of age. More than one joint may be affected. In the acute stage, the joints are not grossly enlarged but they become extended with pus later. The lambs are dull and unthrifty, some showing signs of secondary

pneumonia or meningitis (Angus, 1991). The majority of affected lambs are less than two weeks old, with most cases occurring on day four and five after birth (Tømmerberg et al., 2017). This implies that the lambs acquire the infection at, or shortly after birth.

The navels are frequently listed as the most likely portal of entry for SDSD in the literature (Angus, 1991; Craig et al., 2015). This assumption is largely based on the young age of affected lambs, and the study of Cornell and Glover (1925) who found streptococci in navels of affected lambs from two of three investigated flocks. Blakemore (1939) aimed to induce streptococcal arthritis by soaking the navels of newborn lambs with a streptococcal suspension, but was not able to induce systemic infection. In a larger study, from an unknown number of flocks, Watkins and Sharp (1998) found signs of navel infections in 18/112 (16%) of the lambs with SDSD-arthritis. In all the studies, the authors commented that a considerable proportion of lambs had neither histological nor bacteriological evidence of an ascending infection of the umbilical vessels, and proposed that these lambs were probably infected via another, unknown route. Others have suggested that routes of infection can include *per os* with milk from ewes with mastitis (Lacasta et al., 2008), or contaminated stomach tubes (Anonymous, 2007). Wounds from tail docking, neutering or ear tagging (Anonymous, 2008, 2011), and injection sites (Swinson, 2021) have also been suggested as ports of entry.

The mechanism for how SDSD targets the joints in lambs following bacteraemia is unknown. Experimental studies from the 1940s hypothesized that it is related to the movement or pressure in joints. Blakemore et al. (1941) tested this hypothesis by injecting SDSD intravenously in a lamb fixated in a sac to prevent movement. The lamb nevertheless developed SDSD-arthritis in one joint, and the authors concluded that poor fixation might have been the problem. Other studies have addressed how pyogenic streptococci target joints in other species. For SDSE-arthritis in humans, it has been suggested that "*SDSE traits conferring a tropism for bone and joint are probably unintended side effects from a bacterial point of view, and rather reflect adaptions to other superficial tissues normally colonized and infected by SDSE*" (Oppegaard et al., 2018).

Cases of infectious arthritis in lambs can be sporadic or part of an outbreak. Sporadic cases may have a wide range of bacterial causes. In an outbreak, many cases with similar clinical manifestations occur within a short period. Diagnostic investigations will reveal the same bacterial pathogen from the cases. There is no clear cut-off or defined percentage of lambs with infectious arthritis in a flock to call it an outbreak,

but Rutherford et al (2014) proposed that when at least two percent are affected, it could be defined as an outbreak of infectious arthritis.

Outbreaks of SDSD arthritis are characterised by a sudden onset of acute lameness and deaths, in young lambs, and a rapid within-flock spread (Angus, 1991). This differs from outbreaks caused by *E. rhusiopathiae*, which generally affects older lambs, or *S. aureus*, which is most commonly a secondary infection in tick pyaemia in lambs on pasture (Craig et al., 2015; Ersdal et al., 2015). In any case, the bacteriological diagnosis should ideally be confirmed by bacterial culture of joint aspirates.

Farmers experiencing outbreaks of infectious arthritis in their flocks report that up to 40% of the lambs may be affected (Tømmerberg et al., 2017), and the mortality may range from 0 to 75% (Rutherford et al., 2015). Field experience from Norway shows that the outbreaks lead to a mass treatment with antimicrobials, and a significant extra workload for farmers during lambing. Hence, the outbreaks present a serious negative impact on both animal welfare and the farmer's well-being and economy.

Little is known about risk factors and relevant preventive measures for outbreaks of arthritis caused by SDSD in lambs. Based on previous studies and experience from the field, the Norwegian sheep health service has recommended general measures like improved hygiene in lambing pens and when providing lambing assistance, delayed ear-tagging, disinfection of navels, and reducing stocking density. An experimental whole-cell vaccine with inactivated SDSD has also been used. Ensuring adequate intake of colostrum by lambs has also been a general advice. The recommended treatment of lambs with signs of streptococcal arthritis in Norway is penicillin for a minimum of five days combined with non-steroidal anti-inflammatory drugs (Statens Legemiddelverk, 2012).

5.2.3 Mastitis in cows

Mastitis refers to inflammation of the mammary gland and is most often caused by IMI by bacteria. Mastitis is the most prevalent and costly animal disease affecting the dairy industry worldwide and is a major reason for the use of antimicrobials in the dairy production (Barlow, 2011; Ruegg, 2017). In 2020, 14% of Norwegian dairy cows had a recorded mastitis treatment during lactation (TINE, 2020).

Mastitis may present as subclinical or clinical. In subclinical cases, there are no clinical signs or visual changes in the milk, and diagnostic testing is necessary for detection.

The most widely used tests for detecting subclinical mastitis relate to the somatic cell count (SCC) of milk. Clinical mastitis, on the other hand, implies clinical signs in the animal or visual changes of the milk. It is classified according to disease severity as mild (abnormal milk only), moderate (abnormal milk and a mild/moderate swelling/pain), or severe, with abnormal milk, swelling of the udder, heat, pain, and sometimes fever, lethargy and anorexia (IDF, 2011).

Together with *S. aureus*, *E. coli* and *S. uberis*, SDSD is considered a major udder pathogen in industrialized dairy production because of its ability to cause acute clinical mastitis and induce a strong inflammatory response in the udder. In Norway and Sweden, SDSD is reported to be among the three most common causes of clinical mastitis, following *S. aureus* and *E. coli* (Duse et al., 2021; Ericsson Unnerstad et al., 2009; TINE, 2020). In other countries, *S. uberis* is a more common cause of mastitis than SDSD, whilst *S. agalactiae* dominates in the African and South American continents (Kabelitz et al., 2021). In a screening of bulk milk in 2013, with testing of milk samples from 9398 Norwegian dairy herds, SDSD was detected in of 41% of the farms, similar to the prevalence of *S. aureus* (42%) (not published).

Subclinical mastitis caused by SDSD is characterized by a significant increase in SCC (Heikkilä et al., 2018; Whist et al., 2007a) and an exceptionally high bacterial shedding rate (Hamel et al., 2021). Hence, the subclinical infections significantly impact milk quality, and shedding rates may impact the efficiency of transmission.

Despite its name (*dysgalactiae*) the effects of a subclinical mastitis caused by SDSD on milk production have not been well studied. Whist et al (2007a) found that SDSD-positive primiparous or multiparous cows produced 334 and 246 kg less milk, respectively, during a 305-day lactation period, compared to culture-negative cows. They also reported that first-parity cows with an IMI caused by SDSD at the beginning of their first lactation had an increased risk of clinical mastitis and culling later in the lactation.

Clinical mastitis caused by SDSD may range from mild to severe. The appearance of the milk ranges from watery to clotted or pus-like (Figure 3). Clinically, SDSD-mastitis cannot be distinguished from mastitis caused by other major udder pathogens, and the diagnosis must be based on bacterial culture or qPCR of milk. SDSD is also frequently isolated together with *Trueperella pyogenes* in cows with the summer mastitis/dry cow mastitis complex and in heifer mastitis (Madsen et al., 1990; Schwan and Holmberg, 1979).

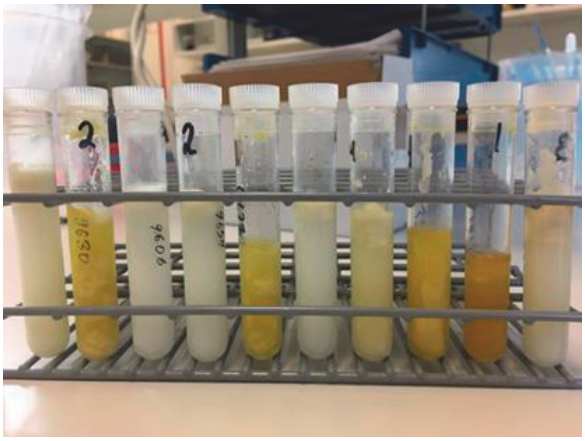


Figure 3: Milk from cows with intramammary infections caused by Streptococcus dysgalactiae *subspecies* dysgalactiae. *Photo: Marit Smistad*

In a retrospective Finnish study utilizing milk samples submitted to the routine mastitis diagnostics, 22% of the cows with SDSD-IMI had clinical mastitis (Heikkilä et al., 2018). In Norway, 28% of the cows with SDSD-IMI had clinical signs or received treatment during lactation, which indicates clinical mastitis (TINE, 2020). Depending on the severity of the clinical signs and the stage of lactation, a case of clinical SDSD mastitis may have long-term consequences for the cow. Heikkilä et al. (2018) found an average loss in daily milk yield of 2 kg and a 305-day lactational loss of 6.4% if the cow had clinical mastitis caused by SDSD before peak lactation.

5.2.3.1 Prevention of mastitis

Prevention of mastitis must take the multifactorial nature of the disease into account, including the many different causative pathogens. The 10-point plan for mastitis prevention, which is implemented in many countries, includes maintaining good barn hygiene, optimizing the milking technique, use of post-milking teat disinfection and dry cow management such as dry cow therapy, and proper hygiene in the dry cow department (NMC, 2016). Measures to prevent IMI will depend on whether the dominating udder pathogens in a herd have their main reservoir in infected udders (contagious/cow-adapted) or in the environment (environmental), because the measures must target relevant reservoirs.

Due to the conflicting results regarding the epidemiology of SDSD in dairy herds (contagious *vs*. environmental), there has been uncertainty regarding the most effective preventive measures against SDSD-IMI. In Norway, the recommendations have been similar to those for contagious udder pathogens, i.e. targeting infected udders, dry cow treatment, post-milking teat disinfection, and culling chronically infected cows. However, control of SDSD-IMI is often unsuccessful, and Whist et al. (2007b) failed to reduce herd prevalence of SDSD-IMI using measures including selective dry cow treatment. Despite targeted advice to Norwegian dairy farmers, the proportion of SDSD-positive milk samples has increased, from 7% in 2006 to 13% in 2019 (TINE, 2020).

Few studies have investigated pathogen-specific risk factors for IMI caused by SDSD. At herd level, a Dutch study from 1999 found associations between SDSD-IMI and poor milking technique, and also a correlation with a high prevalence of *S. aureus*-IMI (Barkema et al., 1999). This could imply that SDSD has common risk factors with *S. aureus*. A study from Finland found an increased risk of SDSD-IMI in herds milked in milking parlour, compared to AMS and pipeline milking (Taponen et al., 2017). On cow level, teat lesions were associated with an increased risk for SDSD-IMI (Ericsson Unnerstad et al., 2009).

5.2.4 Bacterial typing to investigate epidemiology

Typing of bacteria allows differentiation beyond the species and subspecies level. Identification of close genetic relatedness between bacteria is essential in epidemiological studies to identify transmission routes and infection sources.

Typing methods can be phenotypic or genotypic. Phenotypic methods detect variability in bacterial characteristics that result from the expression of genes, e.g. antimicrobial susceptibility, or the ability to ferment sugars. Serotyping identifying variable surface proteins or carbohydrates (polysaccharides), including capsule typing for *S. agalactiae* and M-typing typing for SDSE and *S. pyogenes* have previously been the gold standard for several streptococci (Fischetti, 1989) but the M-protein has not previously been identified in SDSD.

So far, most studies inferring genetic relationships among SDSD-isolates have utilized pulsed-field gel electrophoresis (PFGE) (Baseggio et al., 1997; Lundberg et al., 2014; Wente and Krömker, 2020). This gel-based genotyping relies on whole genome digestion with a restriction enzyme followed by the separation of the fragments in an agarose gel. It is relatively easy to perform but has the disadvantage of being time-

consuming and laborious and has a relatively low resolution. Furthermore, the results are difficult to compare between laboratories and studies, and the interpretation of banding patterns may vary between investigators (Zadoks and Schukken, 2006).

Gel-based methods are increasingly replaced by sequence-based methods, like multi-locus sequence typing (MLST). MLST is based on the sequencing of seven housekeeping genes, and the MLST profile assigns each unique combination of these sequences to a sequence type (ST). It is highly reproducible, making comparisons between labs and studies possible (Larsen et al., 2012). The MLST scheme used for SDSD is the same used for SDSE (McMillan et al., 2010). As of today, none of the STs overlaps between the two subspecies (www.pubMLST.org, accessed June 1st, 2022).

Whole genome sequencing (WGS) is the process of determining the whole DNA sequence of an organism. As the WGS cost continues to decrease, currently below the price of traditional MLST, it has become more available in research and routine diagnostics and typing bacteria directly from WGS data has become routine.

5.2.5 Whole genome sequencing

5.2.5.1 Sequencing technologies

Sequencing has become an essential tool in modern bacteriology. Technological developments have increased the speed and volume of data generated. Whilst first generation sequencing methods (e.g. Sanger sequencing) only sequence a single DNA-fragment at a time, next generation sequencing methods (NGS) (high-throughput sequencing) provide a massive, parallel sequencing of fragments simultaneously.

Next generation sequencing technologies are divided into short read sequencing (second-generation sequencing), and long read sequencing (third-generation sequencing). Illumina sequencing, an example of the former, is the most widely applied sequencing technology in bacteriology today (Segerman, 2020). Different Illumina platforms (e.g. MiSeq, HiSeq), provide different outputs with a maximum length of a few hundred base pairs.

Third generation sequencing technologies, for example Nanopore sequencing, are capable of reading longer lengths, and the technology is based on real-time identification of DNA bases by measuring changes in electric conductivity as DNA strands pass through a biological pore (Jain et al., 2016). Third generation sequencing

can be used to screen environmental and clinical samples for microorganisms without culturing, and can be used in metagenomic research and diagnostics. At present, a disadvantage of this technology is lower accuracy compared to Illumina sequencing (Jain et al., 2016; Segerman, 2020).

The choice of sequencing technology depends on the research questions and resources. Generally, higher discriminatory power, speed and ease of use are associated with a higher cost.

5.2.5.2 Bioinformatics

Data from WGS provide a wide range of opportunities for use, including investigations of phylogenetic relationships between microorganisms, source tracing or search for virulence genes. Common to all high-throughput sequencing methods is the need for bioinformatics analyses. Sequence data from Illumina Miseq, raw reads (50-300 bp), can be assembled to longer sequences (contigs) without a reference genome (*de novo* assembly) or by mapping to a reference genome. Gene prediction (e.g. MLST, virulence genes, antimicrobial resistance (AMR) genes) can be done using contigs as input in bioinformatic tools, whilst detailed phylogeny is often performed by comparing to a reference genome.

Phylogenetic investigations of bacteria are used to identify clusters of closely related bacterial isolates, e.g. identification of outbreak strains. It may also be used in source tracing, where the similarity between two isolates can inform the likelihood that the two isolates are connected. For example, if two cows in the same herd have an IMI caused by the same strain, the infections may result from contagious transmission or a common source. A common approach is to determine the number of nucleotide differences (single nucleotide polymorphisms, SNPs) between two genomes. However, the interpretation of SNP distances must be put into context with epidemiological information and the population structure of the bacterial species (Pightling et al., 2018). The relationship between sequenced isolates is often visualized in phylogenetic trees, where the nodes represent the hypothetical common ancestor, which is inferred based on the descendants (Lemey et al., 2009).

Phylogeny is also used to determine a bacterial species' population structure. Bacterial populations may be clonal or heterogeneous depending on a bacterium's ability to acquire exogenous DNA, the rate of recombination and mutation, the selection pressure and the demographic history (Andam et al., 2017). The bacterial

population structure will impact the questions that can be addressed by molecular epidemiological studies (Spratt and Maiden, 1999).

Other important uses of WGS data include the identification of antimicrobial resistance genes. The genotypic resistance profile generally has a good correlation with the conventional phenotypic methods for many bacterial species (Feldgarden et al., 2019). Finally, information on genes possibly involved in virulence and host adaption may also be extracted from WGS data.

5.2.6 Pathogenesis and involved virulence factors

Bacterial traits that are involved in disease development are termed virulence factors. The severity of an infection is dependent on a balance between the virulence of the pathogen and the resistance of the host. Investigation of bacterial virulence factors may provide important insight into bacterial pathogenesis, including 1) entry into the host with evasion of host primary defences, 2) adhesion of the microorganism to the host cell, 3) propagation of the organism, 4) damage to host cell by toxins or inflammatory response, and 5) evasion of host secondary defences. A search for genes encoding known virulence factors in the genome may provide indications of genetic regions putatively involved in the development of disease.

Studies of SDSD in bovine cell lines have shown that it adheres to mammary epithelial cells (Calvinho and Oliver, 1998a), and can survive intracellularly without destroying the cells (Calvinho and Oliver, 1998b), which might be of relevance in the development of persistent IMI. However, SDSD can also affect the secretory function of the udder epithelial cells (Almeida and Oliver, 1995), a mechanism possibly involved in reduced production following a SDSD-IMI. Invasion into epithelial cells could result in protection from the host's immune system and antimicrobial agents, or alternatively, lead to cell destruction and contribute to tissue invasion (Calvinho and Oliver, 1998b).

Bacterial surface proteins may interact with host-derived proteins, such as albumin and immunoglobulins, and be involved in immune evasion (Calvinho et al., 1998; Kabelitz et al., 2021). Toxins and superantigens, associated with excessive induction of the immune response and streptococcal shock syndrome, have been identified in SDSD-isolates from bovine mastitis (Rato et al., 2011), but associations between superantigens and udder inflammation are unclear. Furthermore, some authors suggested production of hyaluronidase and fibrinolysin to promote dissemination

into host tissue (Sting et al., 1990; Vandamme et al., 1996). Potential virulence mechanisms of SDSD in bovine mastitis are summarized in Figure 4.

Although the majority of previous studies on virulence of SDSD is associated with mastitis in cattle, the virulence factors may also be of relevance for streptococcal invasive disease in lambs. For example, fibronectin and collagen are common components of both the udder and bone. Hence, the repertoire of adhesins may also be involved in the pathogenesis of joint disease in lambs.

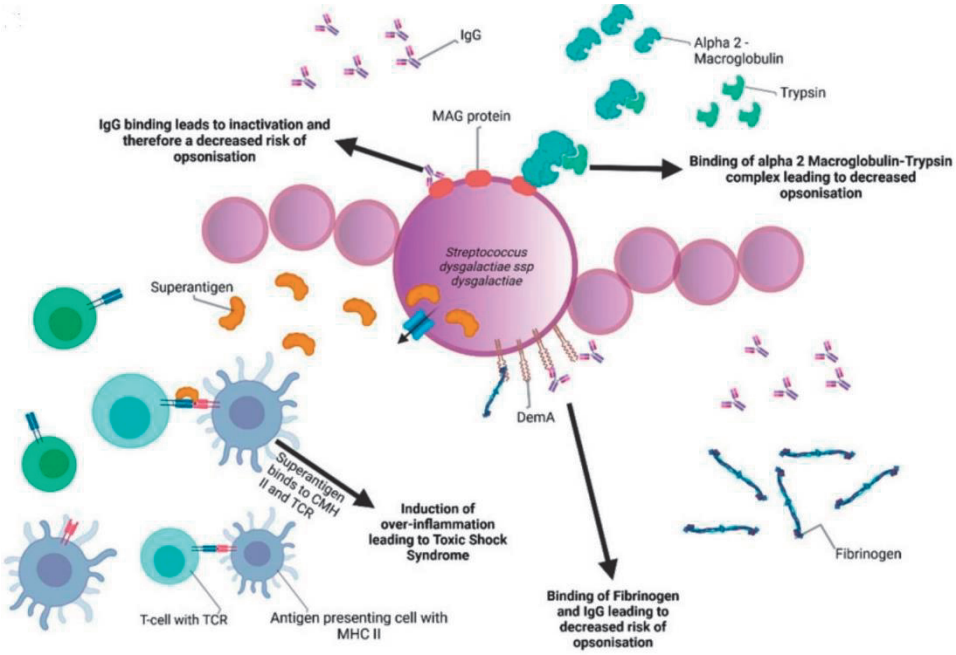


Figure 4: Previously described bovine-associated virulence factors of Streptococcus dysgalactiae *subspecies* dysgalactiae. *The figure is adapted from Kabelitz et al. (2021), printed with permission under the Creative Commons Attribution (CC BY) Licenses https://creativecommons.org/licenses/by/4.0/.*

The different disease outcomes of SDSD infections in lambs (arthritis) compared to in cows (mastitis) may be related to the bacterial strain, the host, or both. Few studies have sequenced SDSD isolates from sheep, and genomes of SDSD isolates from sheep have not been available in open access databases. It is therefore not known whether the SDSD strains that cause outbreaks of infectious arthritis in sheep flocks differ

from strains that cause mastitis in cows. Specific lineages of SDSE have been associated with severe clinical disease manifestations in humans (Oppegaard et al., 2017). Few studies have investigated strain-specific virulence of SDSD. Higgs et al. (1980) challenged quarters of 16 cows with four different strains of SDSD, isolated from cases of clinical mastitis. They found that one strain induced infection, clinical signs and SCC response in all challenged quarters, whilst another strain only exceptionally induced IMI.

5.3 Knowledge gaps and research questions

Streptococcal infections compromise welfare, reduce production, and increase antibiotic usage in food-producing animals. In Norway, the reported incidence of SDSD infections in sheep (outbreaks of infectious arthritis) and dairy cows (mastitis) has increased the last years, in particular in modern management systems. It is not known whether this is related to an altered environment around the animals, facilitating survival of SDSD, reduced host resistance or introduction of new strains. Identification of factors associated with increased risk of disease may help understand the increasing incidence of SDSD infections in Norway, but currently, few studies have investigated risk factors for SDSD infections in sheep and dairy cows.

Targeted prevention of infection requires knowledge about infection dynamics, reservoirs, and transmission routes of the pathogen. The literature on sources of SDSD in sheep flocks and bovine dairy herds is sparse, and no extensive investigations of the reservoir(s) of SDSD have been performed. The primary reservoirs and role of environmental sources in transmission are unknown.

Finally, little has been done with respect to characterising genetic traits of different SDSD strains. We do not know whether genetic factors of SDSD are responsible for the different disease outcomes in sheep and cows, or if certain strains are more contagious or associated with more severe disease. Genomic studies of bacterial isolates coupled with epidemiological information could answer these and other questions relevant to transmission routes and pathogenesis.

The main aim of the thesis work was to identify preventive measures to be able to reduce the burden of SDSD infections in Norwegian sheep flocks and bovine dairy herds. More information on the epidemiology to piece together the chain of infection of SDSD in modern Norwegian sheep farms and bovine dairy herds could help inform measures to improve infection control, reduce disease burden, and ultimately reduce

the need for antimicrobials. To acquire this knowledge, three main research questions were formulated:

1. What are the risk factors for SDSD infections in sheep flocks and bovine dairy herds?

Hypotheses to be tested were:

Factors related to modern management are associated with increased risk of SDSD infections, including new building types, new technology like automatic milking systems in dairy herds, larger flock/herd sizes or higher production.

2. What are the sources and transmission routes of SDSD in sheep flocks and bovine dairy farms?

Examples of hypotheses to be tested:

- High infection pressure, as measured by the proportion of positive samples, is associated with outbreaks of infectious arthritis in sheep flocks
- The vagina of the ewe is a reservoir of SDSD and a potential source of colonization of the lambs during birth
- Oro-faecal transmission of SDSD occurs and contributes to contamination of the environment
- Bacteria from environmental sources transmit to the lamb causing infections through the navel and wounds, and to the cow udder through the teat canal

3. Can genetic characteristics of SDSD strains determine host specificity, environmental persistence, virulence, or contagiousness?

Examples of hypotheses to be tested:

- The different disease pictures in sheep and cows can be explained by strain differences
- A new SDSD strain is contributing to the increased incidence of infectious arthritis in Norwegian sheep flocks, as reported by the sheep industry
- A host species specificity exists among SDSD strains
- Differences in virulence of SDSD may be explained by variation in the presence of virulence genes between strains

6 Materials and methods

This thesis work included a broad repertoire of methods from several research fields, including epidemiology, bacteriology, molecular biology, and genomics. Figure 5 illustrates the elements of work described in this PhD thesis.

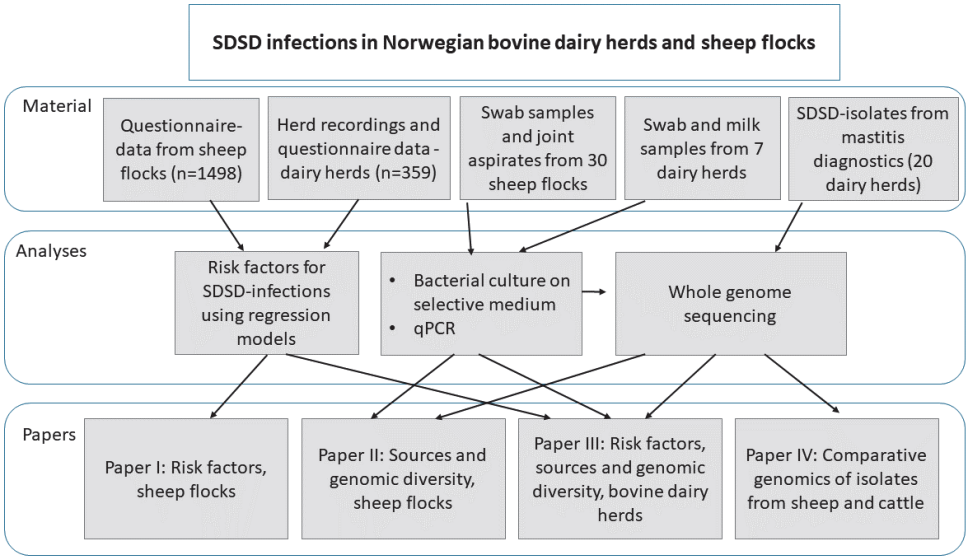


Figure 5: Overview of the materials, analyses and resulting papers in the PhD-work.

6.1 Risk factor studies based on farmer surveys

The risk factor studies aimed to identify characteristics that influence the risk of SDSD infection to provide a knowledge base for advice on preventive measures. Investigation of possible risk factors for outbreaks of SDSD in sheep flocks and for high incidence of SDSD mastitis in dairy farms was based on questionnaires (Papers I and III). The questionnaire data from bovine dairy herds were merged with data from the NHRS (Paper III).

We designed questionnaires in collaboration with the sheep and dairy advisory services, based on literature reviews, empiric knowledge and experience from the field. The questionnaires can be found as Additional file 1 (Paper I, translated version)

and as Appendix I (Paper III, in Norwegian). To ensure clarity, the questionnaires were pre-tested on three sheep farmers and three dairy cow farmers, respectively, and their feedback led to some minor adjustments. To encourage participation, one participant from each study was randomly selected to win an electronic tablet.

In the sheep risk factor study (Paper I), all data were obtained from the questionnaire. The study was a retrospective cross-sectional study, with questions concerning the 2018 lambing season. All farmers reporting to the Norwegian sheep recording system (NSRS) received the questionnaire in March 2019. Respondents who reported that ≥5% of the lambs under four weeks of age were affected by infectious arthritis were defined as case farms.

The dairy herd risk factor study (Paper III) was designed as a retrospective case-control study, where herds with more than 20 lactating cows, and high or low incidence of SDSD-IMI (defined below), were pre-defined as cases or controls, respectively, and selected to receive the questionnaire. The incidence of SDSD-IMI was estimated from available data from the routine mastitis diagnostics in 2017 and 2018 as recorded in the NHRS. The 90th percentile, herds with 10% highest proportion of SDSD-IMI was used as cut-off to define a high proportion of SDSD-IMI. The 10th percentile, herds with 10% lowest proportion of SDSD-IMI, was used as cut-off for control herds. However, the 10th percentile were all herds with 0% SDSD-IMI cases, and therefore, all herds with no SDSD-IMI detected (39%) were defined as controls. Data on e.g. production, herd size, and barn type were retrieved from the NHRS.

In both risk factor studies; multivariable logistic regression models were used to evaluate the association with the binary outcome and potential explanatory variables (Papers I and III).

6.2 Herd visits and sample collection

Over the study period for this PhD work, we purposively selected and visited 30 sheep flocks (Paper II) and seven bovine dairy herds (Paper III) for sampling. The purpose was to investigate potential sources or reservoirs of SDSD. Sheep flocks were visited either by private veterinary practitioners or by members (veterinarians) of the study group. Members of the project group, always including the PhD-candidate, visited all bovine herds. The sheep flocks were visited during the lambing seasons of 2019 and 2020, and during the autumn of 2019. The bovine dairy herds were visited once during the winter (January to March) of 2020.

6.2.1 Selection and recruitment of flocks and herds

6.2.1.1 Sheep flocks

We aimed to include flocks with outbreaks of infectious arthritis and healthy control flocks. To recruit the outbreak flocks, we e-mailed information about the project, before the lambing season in 2019, to 52 veterinary practitioners known to work with sheep. We also posted information on a Facebook group for Norwegian production animal veterinarians (with about 2000 members) and on the Norwegian Meat and Poultry Research Centre website. Veterinarians were encouraged to contact the project group if they came across sheep flocks with an outbreak of arthritis in young lambs. The project would compensate the veterinarian economically for their time, supply sampling instructions, and sampling materials and pay the cost of express postage. The farmers received information about the project, the estimated time, the type of samples and that they would get free analyses and receive results from the bacterial analyses (bacterial culture of joint aspirates). To obtain farmer consent, farmers of outbreak flocks received the information verbally due to time constraints, while farmers of control flocks received written information. All farmers consented to participation.

In some areas, the veterinarians expected outbreaks of infectious arthritis due to a previous history of outbreaks, and they were sent sampling equipment and instructions prior to the lambing season. In other cases, veterinarians contacted us on their way to farms experiencing an outbreak. In the latter case, they received instructions by e-mail and had to utilize the sampling equipment already available in their car. In these "acute" sampling occasions, the adherence to the protocol varied, probably mainly due to shortage of time and limited number of swabs available. Time constraints for veterinary practitioners during a busy lambing season also made control flock visits difficult for some of the veterinarians. We asked veterinarians who visited an outbreak flock to also visit a non-outbreak flock; eight of the 13 participating veterinarians were able to do this. In addition to the outbreak flocks and control flocks, some veterinarians visited flocks with a history of outbreaks before 2019. In total, farmers of 30 sheep flocks consented to participate, of which 11 had an outbreak of infectious arthritis in their flock during the season of sampling and the remaining 19 were non-outbreak flocks.

6.2.1.2 Bovine dairy herds

The intention at the start of this project was to perform a longitudinal study in four to eight bovine dairy herds visiting them four times each over one year. We used the results of milk samples submitted to the TINE mastitis laboratory in Molde to purposively identify herds with SDSD as a "common and recent" finding in milk samples. Because of significant differences between farms with respect to routines for submitting milk samples for bacteriological analyses it was not possible to define an exact cut-off for "common finding", but the selected herds should have at least two to three detections of SDSD in different cows in the last two months.

Two herds were selected for pilot testing of the protocol, and both were visited twice. However, SDSD was detected in very few samples, and it appeared that the detection of SDSD in several milk samples before the first visit had led to measures to reduce it, mainly through antimicrobial treatment of the cows. Because of this, the study was altered to a case-control study in 20 bovine dairy herds; ten with a high prevalence of SDSD-IMI (cases) and ten with good udder health (controls). Based on empirical experience with dairy herds with dominance of streptococcal IMI, we also altered the selection strategy and decided to use the bulk milk SCC as an indicator to find herds with an ongoing udder health problem irrespective of whether they had submitted milk samples for diagnostic testing or not. We contacted and recruited farmers that according to NHRS data recently had experienced a sudden increase in bulk milk SCC, e.g. an increase from 120.000 to 200.000 cells/mL. Based on the results from the risk factor study, we also decided to visit only herds housed in freestalls.

Farmers were contacted by telephone, informed about the project, the estimated time the visit would take and the type of samples to be collected. They were also informed that milk samples analyses would be free of charge and that they would get access to the results. All farmers agreed to participate.

We continued to identify and contact farmers based on this strategy until March 2020, when we had to end the herd visits due to the Covid-19 pandemic. In addition, the laboratory at the Norwegian Veterinary Institute (NVI) was closed for the majority of research activities during most of 2020, also due to the pandemic. For this reason, we were only able to visit seven farms in total, and only farms suspected to have a problem with SDSD-IMI had been visited. The number of cows with SDSD-IMI, out of the sampled cows, in each of the seven farms varied from one to four (10-40%); the farms could therefore neither be classified as cases nor controls. The study, therefore,

became a descriptive study of seven herds with a relatively low prevalence of SDSD-IMI, and not a case-control study.

6.2.2 Herd and flock visits

6.2.2.1 Registrations

During visits to the sheep flocks, the veterinarians used the provided forms to collect the following information: flock size, breed, previous history of outbreaks, housing type (including flooring), dates for lambing period, routines for lambing, ear tagging, navel treatment, feeding practices, as well as the veterinarians' subjective view of hygiene in the farm. The farmer recorded the number of lambs affected by arthritis and reported this to the project when the lambing season ended.

At the visits to the bovine dairy farms, we recorded information on housing and the environment (temperature, humidity). We interviewed the farmer regarding, e.g. cleaning procedures/service protocol for the milking system, routines for dry-off, calf feeding, and teat disinfection. We performed wound scoring of at least 20 cows.

6.2.2.2 Sampling regime

We developed the sampling protocols based on pilot investigations and previously published studies. However, previous investigations had yielded low rates of isolation by bacterial culture, and the investigations were therefore largely explorative. A purposive sampling was done combining the knowledge from previous studies on sources of SDSD and other streptococci and pilot investigations using culturing only for detection. Appendix II provides the sampling protocols for sheep flocks and bovine dairy herds. The upper limit of samples per farm visit was restricted by laboratory capacity, as the culturing procedure was laborious.

Pilot investigations in sheep flocks experiencing outbreaks of SDSD-arthritis in lambs were performed in 2018 using bacteriological culturing for detection. SDSD was not detected in environmental samples in those investigations. This influenced the decision in this study to collect a relatively limited number of samples from the environment focusing to a greater extent on the animals as a source.

The most comprehensive sampling in sheep flocks was performed during the lambing season in 2019 with samples obtained from joints affected with arthritis, skin, ear tag lesions, navels, tonsils, and rectum of the lambs and the vagina, rectum, skin, nose,

and tonsils of ewes (Paper II, table 3). For the purpose of diagnostics, the visiting veterinarians were encouraged to obtain joint aspirates from one swollen joint of lambs with suspected infectious arthritis. Joints from clinically unaffected lambs were not sampled. In outbreak flocks, we selected affected lambs and their mothers for sampling. In non-outbreak flocks, we sampled four arbitrarily selected pairs of ewes and their lambs, when possible, from different pens.

After the sheep were housed during the fall of 2019, ten flocks were visited to collect swabs from body sites of ewes and 6-month-old lambs to identify reservoirs of SDSD outside the lambing season. When the PCR method became available at the lab in fall 2019, the sampling protocol for the 2020-lambing season was adjusted to include more environmental samples and did not include tonsil, rectum, and vaginal samples (Paper II).

In the bovine dairy herds (n=7), the sampling regime included milk samples, animal body sites, the freestall environment, and the milking machine. We selected the ten cows with the highest SCC at the last milk recording before the visit for sampling. From these ten cows, we obtained quarter milk samples by standard aseptic technique and samples from body sites (Paper III).

6.2.2.3 Ethical considerations

The sampling performed in this study with swab sampling of animals and collection of milk did not require ethical approval in Norway (Norwegian Ministry of Agriculture and Food, 2015). Informed consent was obtained from all participants (farmers) in the study, including the risk factor studies, and the farm visits. The veterinarians obtained joint aspirates from lambs with suspected infectious arthritis, as a part of a normal diagnostic evaluation of an outbreak. This procedure is invasive, but was considered a non-experimental clinical procedure, and did not require ethical approval. No joint samples were taken from healthy lambs. The farms included in the study operated under the regulations of the Norwegian Food Safety Authority (Norwegian Ministry of Agriculture and Food, 2004; 2005). Data were collected and stored according to the General Rata Protection Regulation (Regulation (EU), 2016/679).

6.2.3 Bacteriological investigations

We plated the swab samples from sheep flocks on blood agar plates (Oxoid) supplemented with esculin and the Streptococcus supplement SR126 (Oxoid). The

Streptococcus supplement contained oxolinic acid and colistin sulfate and was used to suppress the growth of contaminating bacteria other than streptococci (paper II). The recipe was chosen based on pre-testing of several different media, with different supplements in spiked contaminated samples, performed at the NVI before the PhD-project started.

Despite this semi-selective culturing, the rate of isolation of SDSD during the first two sampling occasions in the sheep flocks was low, and the reading of plates was very laborious. The establishment of a qPCR (Paper II) was therefore an important milestone in the project. The qPCR had a higher sensitivity than culturing (Paper II) and was less laborious. For the third sampling occasion in sheep flocks and all samples in the bovine dairy herds, we therefore used qPCR as a screening tool and only cultured qPCR-positive samples. In addition, we cultured all joint aspirates (Paper II) and milk samples (Paper III) according to the normal diagnostic routines of the NVI.

In Paper II, the association between having experienced an outbreak of infectious arthritis (outbreak flock vs non-outbreak flock) and having a high proportion of SDSD-positive samples at a specific sampling site (above-median vs below-median) was assessed using Fisher's exact test. In the bovine dairy herds, the proportions of positive samples are described, but the sample sizes were too small to make statistical comparisons between sources (Paper III).

6.3 Genome studies

We utilized WGS of SDSD-isolates to identify genotypes associated with infectious arthritis in lambs and mastitis in cows, including whether specific genotypes were associated with increased severity of the disease (Papers II, III and IV). Furthermore, we investigated genomic diversity of isolates within and between sheep flocks and dairy herds to trace sources and improve our understanding of transmission. Paper IV describes the phylogenetic relationship between the genomes and searches for possible virulence genes and genes or genetic regions involved in the host-specificity of SDSD.

6.3.1 Selection of isolates for sequencing

At least one isolate per culture positive sheep flock was sequenced (Paper II). For flocks with many isolates (>5), a selection was made to represent as many sampling sites as possible. All the isolates from the dairy herds were sequenced (Paper III). To

include more geographically spread herds and isolates from clinical mastitis isolates from the routine mastitis diagnostics were included. To investigate the population structure of SDSD (paper IV), we selected one isolate per genotype per herd/flock. All three studies included previously sequenced isolates as reference genomes and for comparison.

The total number of isolates sequenced in the project was 77 from sheep and 89 from cows (Paper II, III and IV). After the papers were published, an additional 46 isolates from sheep and 51 isolates from cows were sequenced to increase the number of flocks and herds. The sheep isolates were obtained from the routine diagnostics at the NVI, from typical cases of outbreaks of infectious arthritis. Furthermore, sheep isolates were available in the NVI biobank from ten of the flocks included in the study described in paper II. These isolates had been collected in 2016, 2017 and 2018 in pilot studies investigating the cause of infectious arthritis in lambs. The additional isolates from cows were obtained from the routine mastitis diagnostics at the TINE mastitis laboratory.

6.3.2 DNA-extraction and whole genome sequencing

DNA extraction and quantification were done at the NVI. Library preparation and Illumina sequencing were done at the Norwegian University of Life Sciences (NMBU). Identical or highly similar methods were used for all sequencing studies (Paper II, III and IV).

6.3.3 MLST and phylogenetic analysis

Assignment of isolates to sequence types (Paper II, III and IV) was done using the draft genome as input in the MLST-tool available online at the Centre for genomic epidemiology at the National Food Institute, Technical University of Denmark. As no scheme is available for SDSD, we used the MLST-scheme of SDSE (McMillian, 2010).

The phylogenetic analysis was done with slightly different bioinformatic tools in the three papers (Papers II, III, IV), but using similar principles. The overall relationship between all isolates in each paper was reconstructed based on a core gene alignment and visualized as phylogenetic trees. We used a maximum likelihood approach to find the tree (model) with best fit, and a bootstrap (Papers II, III, IV) to evaluate the strength/robustness of the estimated phylogenetic relationship in each branch. Thereafter, clades of interest were analysed with deeper resolution excluding recombinant sites. Pairwise core genome single nucleotide polymorphisms (SNPs) were calculated.

7 Results

7.1 Risk factors for SDSD infections in sheep flocks and bovine dairy herds

The risk factor studies for SDSD infections in sheep flocks (Paper I) and bovine dairy herds (Paper III) utilised similar approaches. Data were collected by questionnaires and multivariable logistic regression models were used for analysis of factors associated with SDSD-infections.

For sheep flocks, the outcome variable was defined as outbreak of infectious arthritis (1/0) using an attack rate of ≥5% of young lambs with farmer-reported clinical signs of infectious arthritis to define an outbreak. We assumed, based on findings in pilot studies and the set of inclusion criteria (e.g. less than four weeks of age, outbreak occurring during indoor lambing, systemic signs in addition to joint swellings), that the outbreaks were caused by SDSD. Using this definition, 5.6% of the sheep farmers had experienced an outbreak of infectious arthritis caused by SDSD in their flock in the year of study.

For bovine dairy herds, the outcome was a high or low proportion (1/0) of IMI caused by SDSD. Descriptive statistics in the dairy herds (Paper III) revealed that 79% of the case-herds were stalled in freestalls. Many of the investigated risk factors were relevant only in free-stalls and it was not suitable to compare freestall and tiestall housing in the same model. Therefore, we fitted the model to a subset of observations including only freestalls.

The logistic models in sheep flocks (Paper I) showed that increasing flock size, plastic mesh flooring, and a lambing percentage greater than 200 were associated with an increased risk of outbreaks of infectious arthritis. Furthermore, farmer-observed inflammation in ear-tag wounds was associated with outbreaks.

Bovine dairy herds housed in freestalls with closed flooring were at increased risk of having a high proportion of IMI caused by SDSD, compared to those housed on slatted floor (Paper III). Milking parlour, as compared to automatic milking system, and rubber mats in cubicle bases, compared to mattresses, were significantly associated with a high proportion of SDSD-IMI in cows housed in freestalls.

The risk factor studies were used to inform the investigations of sources and transmission dynamics of SDSD in sheep flocks and dairy herds (Papers II and III).

7.2 Sources of SDSD in sheep flocks and bovine dairy herds

The field studies with sampling in sheep flocks (Paper II) and bovine dairy herds (Paper III) explored the reservoir of SDSD on the animals and in their environment.

7.2.1 Sheep flocks

We visited 30 flocks, 11 of which were affected by an outbreak of infectious arthritis at the time of sampling. SDSD was detected by in joint aspirates from ten of the 11 outbreak flocks. The study thus confirmed that SDSD is the main cause of outbreaks of infectious arthritis in Norwegian sheep flocks.

We found SDSD in 27 of the 30 visited flocks (90%). The farmer-reported attack rate of infectious arthritis at the end of the season ranged from nine to 50% of lambs in the outbreak flocks. The proportions of samples positive for SDSD, by bacterial culture and qPCR, in sheep flocks visited during the lambing season are summarised in Figure 6.

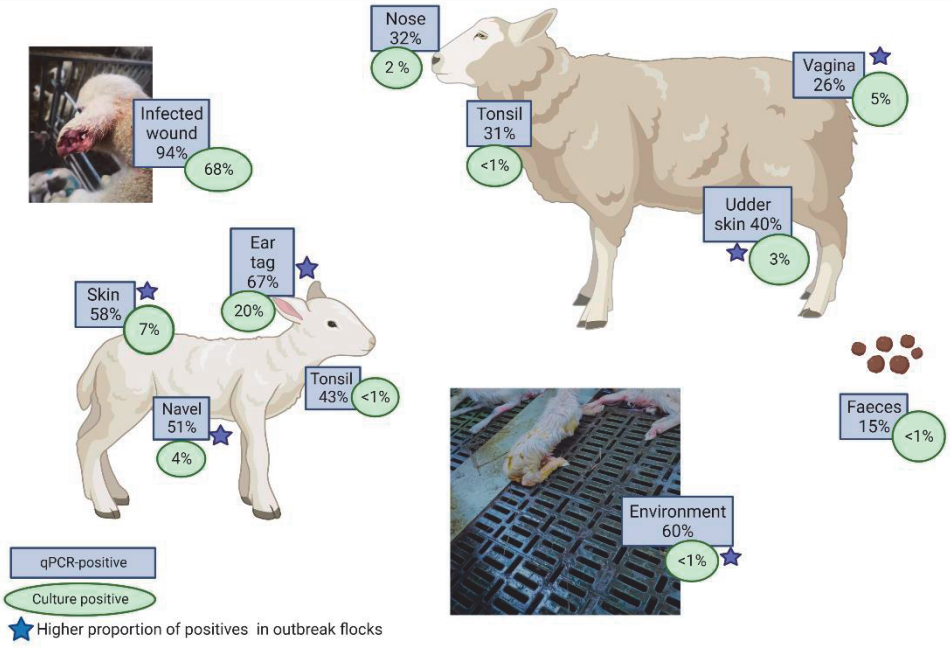


Figure 6: Percentage of samples positive for Streptococcus dysgalactiae *subspecies* dysgalactiae *during lambing season. The figure was created with BioRender.com.*

For ewes, the most commonly positive sampling sites were the skin and mucous membranes. For lambs, the ear tag wounds, skin and navels were most frequently SDSD-positive. In general, detection rates by culture were low, and the difference in bacterial reservoir between outbreak-flocks and non-outbreak flock was not evident. However, when qPCR-results were considered, several sampling sites had significantly higher proportion of positive samples in outbreak flocks during lambing (Figure 6).

At the autumn sampling, there was no significant differences in the proportions of positive samples in flocks that had experienced outbreaks the same year, compared to non-outbreak flocks. At this time, we detected SDSD by qPCR in the ear-tag lesions in 59% of the 6-month-old lambs. Of the udder skin and tonsil samples, 35% and 28% were qPCR-positive, respectively. Vaginal samples were positive in 21% of the ewes, but only 3% of the rectal samples were qPCR positive. Only one tonsil sample was culture positive during the autumn sampling.

The proportion of SDSD-qPCR positive samples on flock-level during the lambing season ranged from 0 to 98% per flock. All outbreak flocks had more than 60% qPCR positive samples. Most non-outbreak flocks (n=11) sampled during lambing had less than 35% qPCR-positive samples. However, four of the 11 non-outbreak flocks had a high proportion (range 50-85%) qPCR-positive samples without experiencing an outbreak.

7.2.2 Bovine dairy herds

We visited seven free-stall bovine dairy herds and collected samples from milk, body sites and the environment (Paper III). The herds had an occurrence of SDSD-IMI ranging from 10-40% of the sampled cows. Figure 7 shows a summary of the proportions of SDSD-positive samples in the bovine dairy herds. The extramammary sampling sites that were most often SDSD-positive were the cow beds, wounds, and the udder skin, whilst mucous surfaces (vagina, nostrils, rectum) were least frequently positive (Figure 7).

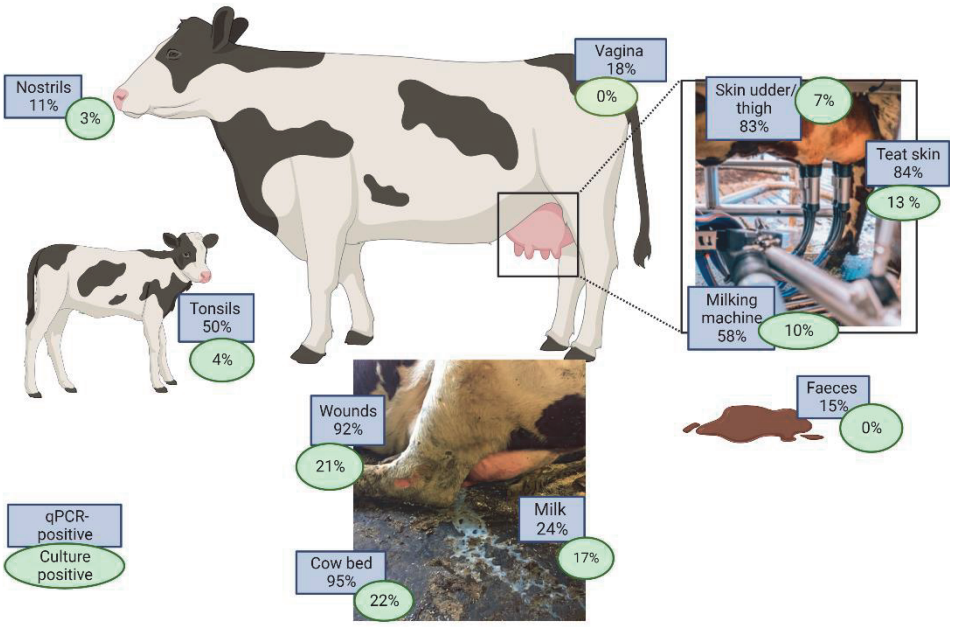


Figure 7: Percentage of samples positive for Streptococcus dysgalactiae *subspecies* dysgalactiae *in bovine dairy herds. The figure was created with BioRender.com.*

7.3 Genomic studies

We utilized WGS to gain an increased understanding of infection dynamics of SDSD in sheep flocks (Paper II) and bovine dairy herds (Paper III). The genomic characteristics of SDSD from cows and sheep were compared to identify genes or genetic regions possibly contributing to host adaption and disease outcome. We also investigated similarities and differences between genomes of SDSD and SDSE from different host species (Paper IV).

7.3.1 Within flock/herd diversity

Isolates from the field investigations were whole genome sequenced to trace sources and assess the genetic variability of SDSD within and between the sheep flocks and dairy herds.

7.3.1.1 Genetic variability in sheep flocks

Among the flocks from which at least four isolates were sequenced (n=8), we found a single ST per sheep flock indicating that within a flock, isolates have high similarity. Strains from joint aspirates in outbreak flocks were also found in other sources in the same farm (Figure 6). Among sheep flocks with at least one SDSD isolate (n=15), STs 454 and 531 dominated, and were found in eight and seven of the flocks, respectively. Seven of the eight flocks from which ST454 was isolated were outbreak flocks, whilst ST531 was found in four outbreak flocks and three non-outbreak flocks. The association between ST (ST454 *vs*. 531) and outbreak (0/1) was, however, not significant (Fisher's exact p=0.10).

The results described above indicated a possible association between ST 454 and outbreaks of infectious arthritis. To further assess this apparent association, we decided to utilise some remaining funding after Paper II was published to sequence isolates from more sheep flocks experiencing outbreaks of infectious arthritis. All additional isolates sequenced belonged to ST531 or 454. In three flocks, isolates from 2016-2018 belonged to ST454, whilst isolates from the same flocks collected during 2019-2020 belonged to ST531.

In total, isolates from 24 sheep flocks were sequenced in the project, among which 15 flocks (62%) and 10 flocks (42%), had ST454 and 531 respectively. Hence, the possible association between ST454 and outbreak was not evident when isolates from more outbreak-flocks were added. From non-outbreak flocks, we found ST454 in one flock, ST531 in three flocks, and ST306 in one flock.

7.3.1.2 Genetic variability in bovine dairy herds

Among the isolates collected during the herd visits, the number of STs per herd ranged from one to three. One strain tended to dominate in each herd, and the dominating strain was found in both in milk (IMI) and extramammary sources. In some cases, we found similar strains of SDSD in milk from multiple cows in the same herd. We found no association between ST and subclinical *vs.* clinical mastitis (Fisher's exact p>0.05), or between ST and sampling site (milk, body sites, environment).

The visited herds were all located in two counties in Eastern Norway, within a two-hour driving distance from the NVI. To assess the genetic variability of SDSD-isolates from different geographic regions, we included SDSD-isolates from the routine

mastitis diagnostics (TINE mastitis laboratory) from 20 different dairy herds from nine of the 11 counties in Norway. Isolates from the 27 herds belonged to a limited number of STs (n=8). ST453 was found in 15 herds (56%) and may represent a bovine-adapted strain. No geographical clustering of STs was observed. Among the 51 isolates (from 51 herds) sequenced after the papers were published, 24 (47%) belonged to ST453, whilst the remaining isolates (range one to five isolates per ST) belonged to STs 301, 302, 306, 454, 460, 529, 531, 532, 629, and 632.

7.3.2 Comparative genomics

To further characterise the genome of SDSD, compare it to SDSE, and investigate any host species adaptations, further genomic investigations were done on selected isolates (Paper IV). Due to the previously determined high similarity between isolates from the same flock/herd (Paper II and III), we selected one isolate per sheep flock (n=19) and one isolate per ST from each dairy herd (n=27) in the comparative study.

Phylogenetically, the SDSD-isolates clustered according to the host species from which it was isolated, with few overlaps. One clade, with isolates belonging to ST531, was shared between sheep and cows.

Several potential virulence genes were ubiquitously present in the genomes from sheep and cow isolates. These were genes mainly involved in adhesion, immune evasion, and dissemination. We found no single gene or genetic region uniquely associated with host species. The SDSD genomes harboured a genetic region that was highly similar to the *srr1* operon previously described in *S. agalactiae*. Putatively, this region is involved in fibrinogen-binding/adhesion also in SDSD. One region of this operon showed two distinct variants (<90% similarity) between isolates from sheep and cows. The role of this region in host specificity and virulence should be further investigated.

Another interesting finding was an *emm*-like gene. The *emm*-gene is used for M-typing of SDSE and *S. pyogenes*, whilst SDSD-isolates have appeared non-typable using the same primers. With few changes in the M-typing primers, we could categorize the SDSD genomes based on the different emm-like types. These groups corresponded with MLST, however, with some greater discrimination. Hence, M-typing may offer a typing method with greater discriminatory power than MLST.

8 Discussion

This work has aimed to enhance our understanding of epidemiology of SDSD, specifically to help explain the chain of infection for this pathogen in Norwegian sheep flocks and bovine dairy herds. Few studies on SDSD had been performed before the project started, and advice for farmers struggling with SDSD infections in their animals were mainly empirical.

The comparative approach of this project, with investigations in sheep and in cattle, was an advantage. The same bacteriological methods, and a similar research approach, was used to investigate the same infectious agent causing different diseases, in two host species kept under relatively different management conditions. The main findings are discussed in detail in the included papers. This section will focus on the comparative aspects of the findings, as well as details less discussed in the papers.

8.1 Interpretation of the results

8.1.1 Risk factors for SDSD infections

The risk factor studies in sheep (Paper I) and dairy cows (Paper III) revealed that several factors related to modern management were associated with SDSD-infections. For example, larger sheep flocks with high lambing percentage had an increased risk of experiencing outbreaks of infectious arthritis. The first sheep flocks with more than 400 ewes were established in Norway after 2010, following production stimulation. These new sheep holdings often have new buildings and are usually well managed with high-quality feeding and high production. However, despite the increasing flock sizes, the Norwegian weather conditions still demand indoor lambing. This is also stimulated by the payment system for lamb meat, which is highest in September, and hence encourages an earlier lambing season. To have more than 1,000 lambs born indoors within a few weeks is a very challenging and intensive system that is likely to be quite different from the outdoor lambing as practiced elsewhere in the world.

In the bovine dairy herds, an important finding was the association of high incidence of SDSD-IMI with freestall housing. We did not find significant risk factors associated with intensification of the dairy production, such as increasing herd size or increasing milk yield. However, although the transition to freestall housing in Norway often

includes an increase in herd size and milk yield to cover the high building costs, the herd sizes are still small, most herds have less than 60 cows, compared to other countries (Barkema et al., 2015). Moreover, the Norwegian bovine milk production is based on a combination breed, the Norwegian red, with a moderate milk yield. Although the structure of the Norwegian milk production is changing, the intensification might be considered modest.

Large flock sizes and freestall housing increase the number of contact possibilities between animals and thus also the opportunities for bacterial transmission. The same is true for systems involving gathering animals on smaller areas, such as central lambing pens in sheep flocks or the waiting area in dairy herds milked in parlours. Questions on the group size of sheep was included in the farmer survey; but the variation within farms was large, and this variable could not be included in the analyses. However, many modern sheep farms have larger group sizes due to the automatic feeding systems (concentrate stations) or round bale feeders. Freestall housing of cows leads to greater direct and indirect contact between cows than in tiestalls.

It is not necessarily only the number of contacts that leads to an increased risk of new infections in larger sheep flocks and freestall dairy herds. Several conditions that could be related to these variables were not measured, including the stocking density, air quality of the barn, hygiene or the barn logistics, which would include the cow traffic system in AMS herds and movement of ewes at lambing. Regarding hygienic conditions and humidity, since electrical cow-trainers are still in use, many Norwegian tiestalls provide a relatively dry and clean environment compared to freestalls.

Interestingly, the type of flooring remained in the final models in both risk factor studies (Figure 8). In the sheep flocks it was plastic mesh flooring, and in the dairy herds the closed flooring. This effect could be related to increased humidity or a build-up of faeces in these systems. Future studies should further investigate the mechanisms behind the impact of flooring.

Figure 8. Sheep stall with plastic mesh flooring (left) and dairy freestall with closed flooring (right). Photo: Marit Smistad

The two risk factor studies indicated that the increased occurrence of SDSD infections in Norwegian cattle and sheep production is likely to be partly associated with structural changes and factors reflecting modernization of management. They also provided a basis to generate hypotheses for the testing in the reservoir investigations. For example, inflammations in ear tag lesions were identified as a risk factor in the sheep study, leading to a specific focus on tag lesions in the field studies. In the dairy herds, we decided to include freestalls only, based on association with freestalls in the risk factor study. To investigate whether the milking system and type of flooring in alleys, which were identified as risk factors, were associated with a potential bacterial reservoir, dairy herds with different flooring types and milking systems were included.

8.1.2 Sources of SDSD in sheep flocks and bovine dairy herds

Following the risk factor studies, sources of SDSD on the animals and in the environment of sheep flocks and bovine dairy herds were studied (Papers II and III). The available literature on SDSD-sources was sparse. Therefore, our investigations were explorative with the aim to identify the main reservoirs of SDSD.

A strength of this project was the combination of bacterial culture and qPCR. The qPCR was developed and validated during the autumn of 2019, after the two first sampling periods in sheep flocks. Ideally, it would have been available before the

study started, but this was not achievable. The sampling protocol in the sheep flocks was therefore designed based on culturing results in pilot studies performed the year before, and the upper limit of collected samples per visit was limited by the laboratory capacity.

Following analyses by qPCR, we revealed patterns differentiating the infection pressure, as measured by the proportion of qPCR-positive samples, in outbreak flocks compared to non-outbreak flocks. Compared to culturing, the sensitivity of qPCR was improved by 83%. The methods correlated well, with consistently lower cycle quantity-values of the culture positive samples. Since qPCR also detects dead bacteria, whilst bacterial culture is dependent on viable bacteria, the true answer to the possible importance of a particular source probably lies somewhere in between the results of qPCR and bacterial culture.

The qPCR results gave an indication of the level of contamination of the environment but could not answer whether the sources are important for transmission. The qPCR-results did serve as a good indicator of the probability to find viable bacteria, and therefore was a convenient screening tool advising on what samples to focus our efforts on to retrieve bacterial isolates. After the first two sampling periods in sheep were analysed by qPCR, we decided to adjust the sampling protocol to include more samples from sheep environment in 2020 (Paper II) and throughout the bovine dairy herd sampling (Paper III).

The combination of results from bacterial culture and qPCR indicated that the bacterium was mainly associated with animal body sites in sheep flocks, particularly wounds, skin, and mucous membranes. A very low proportion of samples from the environment of sheep sheds were culture-positive, indicating that bacterial survival in the environment is low. However, qPCR revealed significant differences in positive samples from the environment between outbreak flocks and non-outbreak flocks, suggesting a more contaminated environment.

Because of the variable compliance with the sampling protocol in sheep flocks at visits during outbreaks, comparison of the proportion of qPCR-positive samples per flock must be done with care. However, it was noteworthy that four non-outbreak flocks had a high proportion (>50%) of qPCR-positive samples, resembling the situation in outbreak flocks. Two of these flocks had an attack rate of arthritis in lambs of one and three percent, just below the cut-off for outbreak flock of 5%. Furthermore, three of these flocks had experienced outbreaks previously, and had introduced several preventive measures. Two of them used an experimental SDSD whole-cell vaccine,

and three had reduced the stocking density. One farmer practiced disinfection of the skin at ear-tagging and had started delaying ear-tagging until the lambs were dry. The fourth flock had not experienced an outbreak previously and had not introduced any preventive measures. This flock was, however, a small flock with all lambings in single pens always rebedded between each lambing. Some inflamed ear-tag wounds were observed in this flock during sampling, which might have contributed to the high proportion of positive samples in that flock. The findings in these flocks indicate that it is possible to avoid an outbreak despite having a significant bacterial reservoir. Our hypothesis that "high infection pressure of SDSD, as measured by a high proportion of positive samples, is associated with outbreaks of infectious arthritis" could only partly be supported.

In the bovine dairy herds, the most frequent SDSD-positive sampling sites were wounds and the skin of the teat and udder. Compared to the sheep flocks, environmental samples from bovine dairy herds were more likely to be culture positive. The bovine freestall environment could be more favourable for the survival of SDSD than the environment in sheep barns. Generally, the bovine freestall environment is more humid than in sheep sheds. In addition, the faecal consistency is looser in dairy cows, which may lead to less hygienic conditions. However, the faecal samples were the least frequently positive in sheep flocks and dairy herds. Pilot investigations in this project also confirmed the poor survival of SDSD in slurry. We could only detect SDSD after one day in SDSD-spiked slurry from a dairy herd kept under aerobic conditions at 20°C (not published). In autoclaved, SDSD-spiked-slurry kept in the fridge, however, we identified SDSD in up to one week indicating that SDSD is a poor competitor with other environmental bacteria. The hypothesis of oro-faecal transmission as an important contribution to contamination of the environment was not confirmed. Poor survival of SDSD in manure contrasts that described for *S. agalactiae* and *S. uberis*, which were relatively frequently isolated from feces (Jørgensen et al., 2016; Lopez-Benavides et al., 2007).

Another plausible explanation for the higher environmental positivity rate in dairy herds may be that cows with SDSD-IMI are more efficient shedders to the environment than lambs with infectious arthritis. In lambs with infections arthritis, the bacteria have no obvious portal of exit, as most lesions are closed. Moreover, the lambs with infectious arthritis tend to become clinically affected, leading to most farmers taking action immediately by segregating and treat diseased animals. Conversely, a cow with subclinical mastitis, can shed millions of bacteria over a longer period without being noticed by the farmer. The shedding rate of SDSD in milk from

cows with SDSD-IMI is known to be very high, and the potential for environmental contamination is likely to be significant.

The cubicle bases were among the most frequently contaminated sites. When cows leak milk, a perfect growth substrate for SDSD is added to the cubicle base, which, as our results suggest, can be contaminated with SDSD from the cow's skin or hock lesions in addition to from infected udders. The percentage of cows leaking milk was initially included as a question in the farmer survey investigating risk factors, but the question was removed from the survey following the pilot test because we received feedback that it was too difficult to assess. Systematic assessments of milk leakage were also beyond the scope of our investigations during the field studies, although efforts were made to check for signs of milk leakage in cubicles. Milk leakage is a known risk factor for mastitis (Schukken et al., 1990; Waage et al., 2001). Whether milk leakage is even more critical concerning SDSD-IMI than for other udder pathogens should be further investigated.

We identified a preference of SDSD to wounds compared to healthy skin in sheep flocks and dairy herds. As Norwegian lambs are not neutered nor tail docked, the most common wounds in young lambs are the ear tag lesions from tagging in the first 2-3 days of life. In bovine dairy herds, hock lesions are common; in fact, a Norwegian study revealed that 60% of the cows in freestalls had hock lesions (Kielland et al., 2009). Our study identified infected ear tag wounds to be a risk factor for outbreaks of infectious arthritis. Furthermore, bacteriological investigations confirmed that ear tag wounds with observed inflammation were often SDSD-positive (94%). In the dairy herds, we scored hock lesions of 20 cows. However, we had too few herds and too little variation in the prevalence of hock lesions between the herds to assess statistical associations between hock lesions and the proportion of SDSD-positive samples. Our risk factor study in dairy herds did identify rubber mats in cow beds as a risk factor compared to mattresses. This may be associated with the fact that rubber mats are more compact and may increase the likelihood of hock lesions (Ekman et al., 2018; Kielland et al., 2009). Based on these observations, it is likely that the wounds serve as multiplication sites and contribute to increased infection pressure of SDSD in a herd or flock.

8.1.3 Transmission of SDSD

The main routes of transmission of SDSD within flocks/herds cannot be firmly determined from this study. Ewes from non-outbreak flocks were found to be colonized, although to a lower extent than ewes in outbreak-flocks. We did not detect

any significant differences between rates of colonization or positive samples between outbreak flocks and non-outbreak flocks in the sampling during the autumn. Due to the relatively high proportion of SDSD-colonized ewes, and the fact that the bacterium appears to be ubiquitously present in sheep flocks, reduction of the ewe reservoir is probably unachievable.

Since 5% and 26% of the vaginal samples from ewes were positive by culture and qPCR, respectively, colonization of the lambs´ skin may occur during birth. We were not able to sample sufficient number of lambs and ewes immediately after birth to support the hypothesis of the vagina as a source of colonization of lambs during birth. Other authors have also hypothesized that vaginal transmission from ewes to lambs during birth and lambing assistance is a potential transmission route (Blakemore et al., 1941; Rutherford et al., 2014). Similarly, transmission of *S. agalactiae* from mother to child during birth in humans, is a known risk factor for neonatal infection in children (Sensini et al., 1997). However, transmission of SDSD to the skin of lambs is likely to happen by many other routes as well, for example during natural interaction between ewes and lambs, like the ewe licking her lamb or when the lamb is searching for the teat. Direct contact with other lambs may also play a role in transmission.

Finally, although probably of less importance, indirect transmission of SDSD from the floor and interior fittings may contribute. The significant effect of plastic mesh flooring in the risk factor study supports the importance of this type of flooring as an indirect transmission route in sheep flocks. Plastic mesh floors have a larger surface area than metal mesh flooring, leaving more fluids and faeces on the surface, and could create more favourable conditions for bacterial survival. Alternatively, the association may be related to altered transmission on these floors. Experience from the field indicates that newborn lambs struggle more to stand up on plastic mesh floors, which may become slippery. It may also be more attractive for a lamb to lie down directly on these floors, compared to metal mesh floorings that feel colder. Unfortunately, only six sheep flocks with plastic mesh flooring were visited in this study, and extended sampling of such floors is needed to assess a role of the floor in transmission.

For dairy cows, the cubicle bases are probably relevant sites of transfer of SDSD between cows. This comes in addition to the transfer of contagious udder pathogens during the process of milking. Overall, 14% and 9% of the environment and body sites samples of the samples from bovine dairy herds were culture positive, respectively. The corresponding percentages in sheep flocks were <1% (environment) and 6%

(body sites). Hence, the environmental transmission route may be more important in bovine dairy herds than in sheep flocks.

8.1.4 Invasion of SDSD

In dairy cows, bacterial invasion of the mammary gland occurs through the teat canal. The main ports of entry of systemic infection of SDSD in lambs, however, are unclear. Our results indicate that the ear tag wounds not only are sites of multiplication of SDSD, but may also serve as infection ports in lambs.

It is also well established that the navel is an entry port in neonatal infectious disease (Watkins, 2007). Cornell and Glover (1925), and Blakemore (1939) studied arthritis in lambs and assumed that the navel was the main port of entry, but were surprised that few lambs had navel lesions, neither grossly nor histologically. In our project, the sampling protocol in sheep flocks included a superficial swabbing of navels only. We found that navels were the second most common SDSD-positive sampling site, after the ear tag wounds.

The oral route of infection was proposed by Lacasta et al. (2008) because they found one lamb with SDSD-arthritis, and isolated SDSD from the milk of the mother. In our study, the tonsils/throat and the rectum were extensively sampled but had a relatively low (tonsils) or very low (faeces) positivity rate for SDSD. We did not collect milk samples from ewes in this project due to findings in a previous study indicating an extremely low prevalence of SDSD in milk from Norwegian ewes, also in flocks with arthritis outbreaks (Tollersrud et al., 2018). We cannot dismiss the possibility that the oral route plays a role but we suggest that wounds and navels are the major ports of infection. The main route of infection in an outbreak of infectious arthritis is probably flock-dependent, and there may be combinations of different routes in flocks, especially in flocks with high infection pressure.

8.1.5 Measures to break the chain of infection

Infection control should aim to break at least one of the links of the chain of infection. Figure 9 summarises the suggested focus on measures to prevent the spread of SDSD infections in sheep flocks and bovine dairy herds.

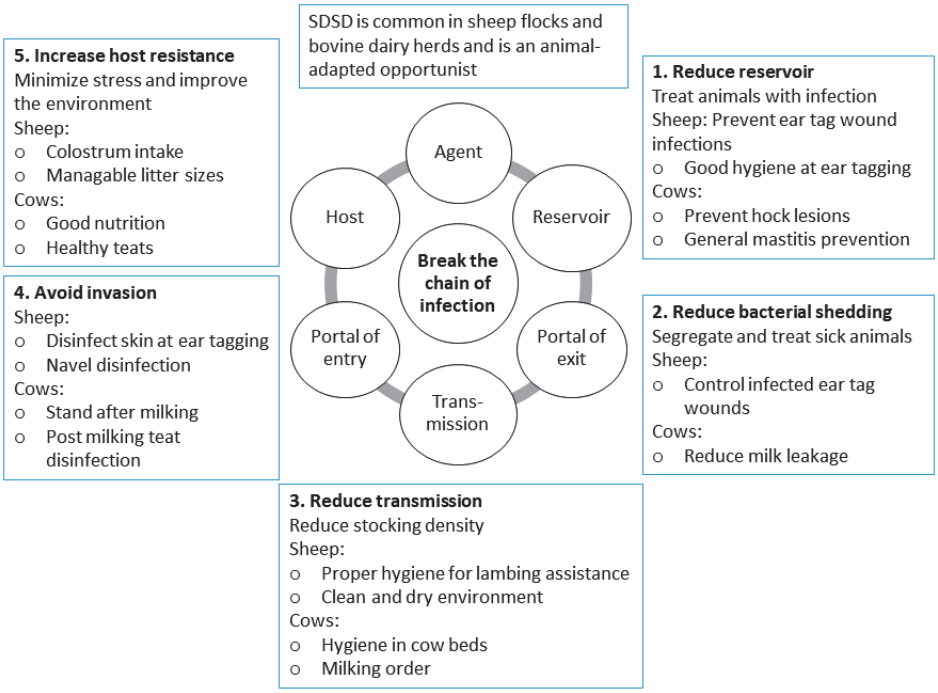


Figure 9: Suggested measures to break the chain of infection of infections caused by Streptococcus dysgalactiae *subspecies* dysgalactiae *(SDSD) in sheep and cattle herds.*

8.1.6 Genome studies

Whole genome sequencing was utilized to investigate host adaption, the within- and between-herd genetic diversity of SDSD, and for source tracing. The diversity within herds/flocks was low for both cattle and sheep, with only one ST identified per sheep flock, and between one and three STs per bovine dairy herd. We identified no more than nine different STs among the isolates described in Paper II and III. Although exceptions existed, the SDSD isolates were phylogenetically delineated according to host species (paper IV) and had a low genetic diversity within a host species (paper II, III and IV), supporting the hypothesis of a degree of host specificity of SDSD.

Three STs were identified among isolates from both sheep and cows (shared STs). The two dominating STs identified in sheep flocks (STs 531, 454), were also identified from cases of bovine IMI, showing that similar bacterial genotypes cause different diseases in different host species. Although this is not evidence to reject the hypothesis of strain differences influencing the different disease picture in sheep and cattle, it suggests that the manifestation of disease as arthritis or mastitis is dependent on the host rather than the strain.

Together, our findings indicate a clonal population structure of SDSD and that the bacterium has existed with the hosts over time. We found no evidence that the increasing incidence of SDSD in Norwegian sheep flocks and bovine dairy herds is due to the introduction of new strains. Instead, we suggest that other predisposing factors including changes in the hosts' susceptibility or alterations to the environment the host lives in, plays a role, as discussed above.

Genetically similar SDSD isolates, defined as the same strain, were found both in association with infection and outside diseased organs. Hence, although we cannot prove the direction of transmission, we believe that the SDSD-positive sampling sites found in the field study are likely to be relevant as bacterial sources for the investigated diseases. For example, all strains identified in milk samples in the dairy herds were also found in the environment and/or on body sites. We also found cases of multiple cows infected with the same strain within herds, indicating contagious transmission. On the other hand, we also found multiple cows in the same herd with SDSD-IMI caused by different strains, showing that the IMIs caused by SDSD are not only a result of contagious transmission. With reference to the epidemiological triad (Figure 1), the herds and flocks with high incidence of SDSD-related disease, do not necessarily have more contagious strains circulating (the agent), but might be exposed to common risk factors (affecting the host resistance or the environment).

ST454 and 531 were the most common STs identified in sheep flocks experiencing outbreaks of infectious arthritis in Norway. The two remaining STs identified were found in one outbreak flock (ST298) and one non-outbreak flock (ST306). From 12 (63%) of the non-outbreak flocks, we did not succeed in culturing isolates, most likely because of a low concentration of viable culturable bacteria, indicating a smaller bacterial reservoir. Based on this it is not possible to describe the genetic diversity of SDSD in healthy sheep flocks, and the question remains whether flocks with outbreaks have a different repertoire of SDSD variants than healthy flocks. Since most non-outbreak flocks were qPCR-positive and culture-negative, the limitation of the investigation of genetic diversity lies in the low sensitivity of culture-dependent methods. Future studies would benefit from culture independent typing methods.

The search for virulence genes (Paper IV) identified several factors associated with adhesion in isolates from sheep and cows. For example, the fibronectin-binding adhesins, *fnbA* and *fnbB,* were found in all included genomes from sheep and cows and approximately 40% of the isolates harboured genes encoding the fibrinogen binding proteins DemA (Paper IV). As both mammary and bone tissue are rich in fibronectin and collagen, these may be important targets for bacterial attachment to

host cells both in sheep and cows. The genetic basis for the tissue tropism for bone and joints has been investigated in SDSE-isolates from humans, but the authors did not identify any single gene to be predictive of clinical outcome (Oppegaard et al., 2018). They proposed, however, that the adhesive properties of SDSE-isolates may have an important role in the arthritogenicity of the bacterium. Possibly, the preference of SDSD to wounds could also be related to adhesins and utilization of fibrinogen, which are important in wound healing. However, the wound predilection may also be related to other factors, such as less competition from other bacteria or beneficial nutritional substrates for SDSD. Future *in vitro* and possibly also *in vivo* studies to investigate the mechanisms of SDSD invasion and tissue tropism are needed to improve our understanding of pathogenesis.

8.2 Methodological considerations

8.2.1 Risk factor studies

Both risk factor studies were based on questionnaire data. Questionnaires have the advantage of being relatively quick and easy to perform and may reach out to many participants. However, some variables can be difficult to assess via questionnaires. The outcome variable in the sheep risk factor study (Paper I), was based on the farmer-reported attack rate of infectious arthritis in the flock the previous lambing season, which may be imprecisely recorded. Furthermore, self-assessment of the farmer's own barn hygiene or other management practices are subjective and subject to bias. Some variables proved to be difficult for the farmer to estimate, like the area per sheep and stocking density in the sheep flocks, and the proportion of cows leaking milk. Our questionnaire-based studies may also be subject to recall bias. For example, the selection of dairy herds was based on data from 2017-2018, whilst the farmers responded to the questionnaire in 2019. Some management practices, for example the use of teat disinfectant, were excluded because it could be difficult for the farmer to recall whether or not teat disinfectant was used in the relevant period, as well as the type of disinfectant.

The farmers that respond to a questionnaire may not be completely representative of the target population. For example, farmers who have experienced a problem related to SDSD infections in their herd or flock may be more inclined to answer. For sheep flocks, we might therefore have overestimated the prevalence of outbreaks of infectious arthritis in Norway. By selecting dairy herds based on results from milk samples submitted for diagnostic testing, we may have an overrepresentation of

farmers that have introduced preventive udder health measures. This may have reduced the ability to detect management practices associated with a high incidence of SDSD-IMI. One example is the use of selective dry cow therapy, which appeared to increase the risk of SDSD-IMI, but was probably rather a consequence of having a high incidence of IMI. The variable was, therefore, not included in the analysis.

In dairy herds, the definition of cases and controls was based on the results of samples submitted to the routine diagnostics, and a calculated proportion of SDSD-IMI among the sampled cows in a herd (Paper III). To define SDSD-case herds, we ranked all the herds submitting milk samples and selected the herds with 10% highest proportion of SDSD-IMI (calculated as described above) as case herds. This proportion will be affected by the sampling strategy of the herd. Moreover, smaller herds are more likely to have a sufficient number of samples and each finding of SDSD will contribute relatively more than in a larger herd. We attempted to reduce this bias by excluding herds with less than 20 cows, and herds that submitted milk samples of less than 5% of the cows. This may have contributed to the lack of effect of herd size in the model.

8.2.2 Sampling

The sample collection in sheep flocks suffering outbreaks of infectious arthritis was done primarily by field veterinarians, during a busy lambing period. Thirteen different veterinarians were involved in the sampling of 30 flocks. Among the participating field veterinarians, the compliance with the sampling protocol and the available sampling equipment varied, especially during acute sampling in outbreak flocks. Another issue was that sampling was done on emergency visits to the farms, and for several flocks there was no time to send sampling equipment to the veterinarians before the visit and commencement of treatment. Lack of swabs was therefore a limiting factor in some flocks. To ensure reasonably equal geographic distribution of outbreak flocks and non-outbreak flocks, the veterinarians visiting an outbreak flock were encouraged to also visit a non-outbreak flock. Eight of the veterinarians that visited an outbreak flock, also visited a non-outbreak flock. Some areas had veterinarians that were especially eager to participate, and this led to a few geographical areas of Norway being overrepresented in the material.

Since we sampled the bovine dairy herds after the sheep flocks, we could utilize the experience from the sheep sampling when designing the dairy herd protocol. For example, a larger proportion of environmental samples were collected in the dairy herds. Furthermore, the upper limit of samples per visit could be increased, because we had developed the qPCR and were able to screen the samples by qPCR before

culturing, thus reducing labour intensity. The sampling in dairy herds was done in farms located within two-hour driving distance from Oslo. We added milk samples from the routine diagnostics from 20 dairy herds across the country to assess the diversity of SDSD-isolates from larger geographic areas, and to validate the relevance of our results in the seven visited herds.

8.2.3 Genome studies

The isolates sequenced in this project were selected to represent different sampling sites and different herds/flocks. However, from some flocks/herds, there were few isolates available, and therefore flocks/herds with many isolates were overrepresented. To assess the genetic diversity of SDSD in sheep and cattle in Norway, isolates from a greater number of herds/flocks would be necessary.

9 Future perspectives

Despite the major importance of SDSD-associated disease in both sheep and cattle, surprisingly few studies had investigated bacterial characteristics, reservoirs, and risk factors prior to this study. Considering this relatively limited knowledge base, we believe that our project has provided significant new knowledge informing preventing measures and also directing future research.

In the questionnaire-based risk factor studies we were not able to evaluate several parameters that might be of relevance for SDSD infections. Future observational studies should include more accurate registrations on stocking density, percentage of leaking cows, the prevalence of hock lesions, hygiene in cow beds, technical function of the milking machine, hygiene practices at milking, prevalence of infected ear tag wounds and humidity. These factors were difficult to measure by questionnaire and were too time-consuming to achieve during farm visits. Future studies should include some of these parameters.

Our study has revealed potential sources of SDSD in sheep flocks and dairy herds, but not the relative significance of each source or the direction of transmission. Our investigations suggest an opportunistic behaviour of SDSD. The bacterium is present in most herds/flocks and has many reservoirs external to the diseased animal, as well as a considerable proportion of healthy animals colonized. The combination of culturing, qPCR and genome sequencing provided a good indicator of the size of the bacterial reservoir indicating the potential infection pressure and also the genetic diversity of SDSD in the visited herds/flocks. It was interesting to see that some sheep flocks had a high proportion of positive samples, indicating a sizable reservoir, without experiencing outbreak. During the autumn, the reservoir in sheep flocks was independent of the outbreak history. Therefore, future studies in sheep flocks should look more into the build-up of the bacterial reservoir and infection pressure. Moreover, we need to explore what are the triggering events leading to an outbreak.

Wounds are probably important when it comes to increasing infection pressure in a herd or flock. In dairy cows, SDSD-infected udders are possibly more important contributors to contamination of the environment. Future investigations can provide an evidence base to confirm or reject these hypotheses by investigating the association between the prevalence of wounds, SDSD-IMI and infection pressure in

the herd or flock. Risk factors for ear tag wound infections in sheep flocks should also be investigated.

The WGS performed in this study was dominated by isolates from outbreak flocks, mainly limited by the low sensitivity of the culturing method. However, the qPCR-results confirmed the presence of SDSD also in non-outbreak flocks. Hence, the picture of the genetic diversity of SDSD is incomplete when utilizing culture-dependent methods. Bacteriological investigations to culture SDSD from a higher number of healthy sheep flocks is advisable. The utilization of culture-independent typing methods could also be relevant. This could be achieved by identification of genetic regions with high variability between sequence types that can be characterised directly from template DNA extracted from environmental samples. Finally, the approximately 250 new SDSD-genomes uploaded to public databases in this project provide opportunities for further bioinformatics investigations.

10 Concluding remarks

Infections caused by *Streptococcus dysgalactiae* subspecies *dysgalactiae* (SDSD) in sheep flocks and bovine dairy herds have been investigated using different methodological approaches, including studying factors associated with the host (risk factors, sources), environment (risk factors, sources), and the pathogen (conventional methods, molecular detection, and whole genome sequencing). Altogether, this project has contributed to explaining some reasons for the increased reports on SDSD-related disease in Norwegian livestock in the past two decades. Some farmers have already reported the project to have supported their work to reduce the disease burden from SDSD (Bergo, 2021).

Modernization of management may alter the host susceptibility or the environment around the animals predisposing them to SDSD infection or increasing the risk of exposure. Several factors associated with modern management were identified as risk factors, namely increased flock size, plastic mesh flooring, higher lambing percentage in sheep flocks (Paper I) and freestall housing in dairy herds (Paper III).

SDSD is commonly present in most visited sheep flocks and dairy herds and our study indicates that it thrives best on the animal, in particular in wounds and the skin. We propose that direct contact is the main route of transmission in sheep flocks (Paper II), whilst the positivity rate of environmental samples in bovine dairy herds indicates that transmission occurs both in the environment and at milking (Paper III). The results also support the hypothesis that ear-tag wounds are likely to be important ports of infection in lambs in addition to navels (Paper II).

Increased virulence of the pathogen itself or the introduction of new, more virulent strains may also explain the increased prevalence of a disease. The genomic investigations revealed host-specific lineages of SDSD, a clonal population structure and a low genetic diversity of strains within and between farms (Paper II, III, IV). We found no evidence of newly introduced, more virulent strains. The close phylogenetic relationship between isolates from epidemiologically independent farms indicates that the bacterium has lived with the hosts over time (Paper IV).

Overall, this work has moved our basic understanding of SDSD infections a step forward. The results of these studies have led to updated advice for Norwegian sheep and dairy farmers, contributing to reduced antimicrobial usage, better animal welfare and improving farmer economy.

11 References

Almeida, R.A., Oliver, S.P., 1995. Invasion of bovine mammary epithelial cells by Streptococcus dysgalactiae. J. Dairy Sci. 78, 1310-1317.

Andam, C., Challagundla, L., Azarian, T., Hanage, W., Robinson, D., 2017. Population structure of pathogenic bacteria. Genetics and Evolution of Infectious Diseases, 51-70.

Angus, K., 1991. Arthritis in lambs and sheep. In Pract. 13, 204-207.

Anonymous, 2007. VLA surveillance report. Vet. Rec. 160, 783-786.

Anonymous, 2008. VLA Surveillance report. Vet. Rec. 158, 639-642.

Anonymous, 2011. VLA Surveillance report. Vet. Rec. 168, 401-403.

Barkema, H., Schukken, Y., Lam, T., Beiboer, M., Benedictus, G., Brand, A., 1999. Management practices associated with the incidence rate of clinical mastitis. J. Dairy Sci. 82, 1643-1654.

Barkema, H.W., von Keyserlingk, M.A., Kastelic, J.P., Lam, T., Luby, C., Roy, J.-P., LeBlanc, S.J., Keefe, G.P., Kelton, D.F., 2015. Invited review: Changes in the dairy industry affecting dairy cattle health and welfare. J. Dairy Sci. 98, 7426-7445.

Barlow, J., 2011. Mastitis therapy and antimicrobial susceptibility: a multispecies review with a focus on antibiotic treatment of mastitis in dairy cattle. J. Mammary Gland Biol. Neoplasia 16, 383-407.

Baseggio, N., Mansell, P.D., Browning, J.W., Browning, G.F., 1997. Strain differentiation of isolates of streptococci from bovine mastitis by pulsed-field gel electrophoresis. Mol. Cell. Probes 11, 349-354.

Bergo, K., 2021. Overcame infectious arhtirits (in Norwegian). [Fikk bukt med leddbetennelsene]. Norsk Landbruk 11-2021, 54-59 https://www.norsklandbruk.no/husdyr/fikk-bukt-med-leddbetennelsene/, accessed August 1st, 2022

Blakemore, F., 1939. Joint-ill (Polyarthritis) of Lambs in East Anglia. Vet. Rec. 51, 1207-1220.

Blakemore, F., Elliott, S., Hart-Mercer, J., 1941. Studies on suppurative polyarthritis (joint-ill) in lambs. The Journal of Pathology and Bacteriology 52, 57-83.

Bramley, A., Hillerton, J., Higgs, T., Hogben, E., 1985. The carriage of summer mastitis pathogens by muscid flies. Br. Vet. J. 141, 618-627.

Bramley, A.J., 1982. Sources of Streptococcus uberis in the dairy herd: I. Isolation from bovine faces and from straw bedding of cattle. J. Dairy Res. 49, 369-373.

Calvinho, L., Almeida, R., Oliver, S., 1998. Potential virulence factors of Streptococcus dysgalactiae associated with bovine mastitis. Vet. Microbiol. 61, 93-110.

Calvinho, L., Oliver, S., 1998a. Factors influencing adherence of Streptococcus dysgalactiae to bovine mammary epithelial cell monolayers. Journal of Veterinary Medicine, Series B 45, 161-170.

Calvinho, L., Oliver, S., 1998b. Invasion and persistence of Streptococcus dysgalactiae within bovine mammary epithelial cells. J. Dairy Sci. 81, 678-686.

Chirico, J., Jonsson, P., Kjellberg, S., Thomas, G., 1997. Summer mastitis experimentally induced by Hydrotaea irritans exposed to bacteria. Med. Vet. Entomol. 11, 187-192.

Cornell, R., Glover, R., 1925. Joint ill in lambs. Vet. Rec. 5, 833-839.

Craig, L.E., Dittmer, K.E., Thompson, K.G., eds. 2015. Bones and joints. Jubb, Kennedy, and Palmer's pathology of domestic animals 1, 16-163.

Cruz Colque, J., Devriese, L., Haesebrouck, F., 1993. Streptococci and enterococci associated with tonsils of cattle. Lett. Appl. Microbiol. 16, 72-74.

Cullen, G., 1966. The ecology of Streptococcus uberis. Br. Vet. J. 122, 333-339.

Daleel, E., Frost, A., 1967. Some observations on the bacterial flora of the bovine tonsil. Br. Vet. J. 123, 232-236.

Diernhofer, K., 1932. Aesulinbouillon as Hilfmittel für die Differenzierung von Euter-and Milchstreptokokken bei Masseuntersuchungen. Milchwirts Forsch 13, 368-374.

Duse, A., Persson-Waller, K., Pedersen, K., 2021. Microbial Aetiology, Antibiotic Susceptibility and Pathogen-Specific Risk Factors for Udder Pathogens from Clinical Mastitis in Dairy Cows. Animals 11, 2113.

Ekman, L., Nyman, A.-K., Landin, H., Persson Waller, K., 2018. Hock lesions in dairy cows in freestall herds: a cross-sectional study of prevalence and risk factors. Acta Vet. Scand. 60, 1-12.

Ericsson Unnerstad, H., Lindberg, A., Waller, K.P., Ekman, T., Artursson, K., Nilsson-Öst, M., Bengtsson, B., 2009. Microbial aetiology of acute clinical mastitis and agent-specific risk factors. Vet. Microbiol. 137, 90-97.

Ersdal, C., Jørgensen, H., Lie, K.-I., 2015. Acute and chronic Erysipelothrix rhusiopathiae infection in lambs. Vet. Pathol. 52, 635-643.

Feldgarden, M., Brover, V., Haft, D.H., Prasad, A.B., Slotta, D.J., Tolstoy, I., Tyson, G.H., Zhao, S., Hsu, C.-H., McDermott, P.F., 2019. Validating the AMRFinder tool and resistance gene database by using antimicrobial resistance genotype-phenotype correlations in a collection of isolates. Antimicrob. Agents Chemother. 63, e00483-00419.

Fischetti, V.A., 1989. Streptococcal M protein: molecular design and biological behavior. Clin. Microbiol. Rev. 2, 285-314.

Fuquay, J.W., McSweeney, P.L., Fox, P.F., 2011. Encyclopedia of dairy sciences. Academic Press.

Hamburger Jr, M., 1947. Transfer of beta hemolytic streptococci by shaking hands. The American Journal of Medicine 2, 23-25.

Hamburger, M., Green, M.J., Hamburger, V.G., 1945. The Problem of the" Dangerous Carrier" of Hemolytic Streptococci: II. Spread of Infection by Individuals with Strongly Positive Nose Cultures Who Expelled Large Numbers of Hemolytic Streptococci. The Journal of infectious diseases 77, 96-108.

Hamel, J., Zhang, Y., Wente, N., Krömker, V., 2021. Heat stress and cow factors affect bacteria shedding pattern from naturally infected mammary gland quarters in dairy cattle. J. Dairy Sci. 104, 786-794.

Heikkilä, A.-M., Liski, E., Pyörälä, S., Taponen, S., 2018. Pathogen-specific production losses in bovine mastitis. J. Dairy Sci. 101, 9493-9504.

Higgs, T., Neave, F., Bramley, A., 1980. Differences in intramammary pathogenicity of four strains of Streptococcus dysgalactiae. J. Med. Microbiol. 13, 393-399.

IDF, 2011. Suggested Interpretation of Mastitis Terminology. Bulletin of the IDF N° 448/ 2011.

Jain, M., Olsen, H.E., Paten, B., Akeson, M., 2016. The Oxford Nanopore MinION: delivery of nanopore sequencing to the genomics community. Genome Biol. 17, 1-11.

Jordal, S., Glambek, M., Oppegaard, O., Kittang, B.R., 2015. New tricks from an old cow: infective endocarditis caused by Streptococcus dysgalactiae subsp. dysgalactiae. J. Clin. Microbiol. 53, 731-734.

Jørgensen, H., Nordstoga, A., Sviland, S., Zadoks, R., Sølverød, L., Kvitle, B., Mørk, T., 2016. Streptococcus agalactiae in the environment of bovine dairy herds–rewriting the textbooks? Vet. Microbiol. 184, 64-72.

Kabelitz, T., Aubry, E., van Vorst, K., Amon, T., Fulde, M., 2021. The Role of Streptococcus spp. in Bovine Mastitis. Microorganisms 9, 1497.

Keefe, G., 2012. Update on control of Staphylococcus aureus and Streptococcus agalactiae for management of mastitis. Veterinary Clinics: Food Animal Practice 28, 203-216.

Kielland, C., Ruud, L., Zanella, A., Østerås, O., 2009. Prevalence and risk factors for skin lesions on legs of dairy cattle housed in freestalls in Norway. J. Dairy Sci. 92, 5487-5496.

Kruze, J., Bramley, A.J., 1982. Sources of Streptococcus uberis in the dairy herd: II. Evidence of colonization of the bovine intestine by Str. uberis. J. Dairy Res. 49, 375-379.

Lacasta, D., Ferrer, L.M., Ramos, J.J., Loste, A., Bueso, J.P., 2008. Digestive pathway of infection in Streptococcus dysgalactiae polyarthritis in lambs. Small Rumin. Res. 78, 202-205.

Lamm, C.G., Ferguson, A., Lehenbauer, T.W., Love, B., 2010. Streptococcal infection in dogs: a retrospective study of 393 cases. Vet. Pathol. 47, 387-395.

Larsen, M.V., Cosentino, S., Rasmussen, S., Friis, C., Hasman, H., Marvig, R.L., Jelsbak, L., Sicheritz-Pontén, T., Ussery, D.W., Aarestrup, F.M., 2012. Multilocus sequence typing of total-genome-sequenced bacteria. J. Clin. Microbiol. 50, 1355-1361.

Lemey, P., Salemi, M., Vandamme, A.-M., 2009. The phylogenetic handbook: a practical approach to phylogenetic analysis and hypothesis testing. Cambridge University Press.

Lemon, H., Loosli, C., Hamburger Jr, M., 1948. The transmission and control of respiratory diseases in army barracks: II. The spread of hemolytic streptococcal infections among enlisted personnel. The Journal of Infectious Diseases, 72-85.

Lopez-Benavides, M., Williamson, J., Pullinger, G., Lacy-Hulbert, S., Cursons, R., Leigh, J., 2007. Field observations on the variation of Streptococcus uberis populations in a pasture-based dairy farm. J. Dairy Sci. 90, 5558-5566.

Lundberg, Å., 2015. Mastitis in Dairy Cows: Genotypes, Spread, and Infection Outcome of Three Important Udder Pathogens. Doctoral Thesis Swedish University of Agricultural Sciences Uppsala, 89

Lundberg, Å., Nyman, A., Unnerstad, H.E., Waller, K.P., 2014. Prevalence of bacterial genotypes and outcome of bovine clinical mastitis due to Streptococcus dysgalactiae and Streptococcus uberis. Acta Vet. Scand. 56, 1-11.

Madsen, M., Sørensen, G.H., Aalbaek, B., 1990. Summer mastitis in heifers: a bacteriological examination of secretions from clinical cases of summer mastitis in Denmark. Vet. Microbiol. 22, 319-328.

McMillan, D.J., Bessen, D.E., Pinho, M., Ford, C., Hall, G.S., Melo-Cristino, J., Ramirez, M., 2010. Population genetics of Streptococcus dysgalactiae subspecies equisimilis reveals widely dispersed clones and extensive recombination. PLoS One 5, e11741.

Miescher, G., Lincke, H., Rinderknecht, P., 1955. Investigation on bacterial desiccation. J. Invest. Dermatol. 24, 293-300.

Neave, F., Dodd, F., Kingwill, R., Westgarth, D., 1969. Control of mastitis in the dairy herd by hygiene and management. J. Dairy Sci. 52, 696-707.

NMC, 2016. http://www.nmconline.org/wp-content/uploads/2016/08/RECOMMENDED-MASTITIS-CONTROL-PROGRAM-International.pdf, accessed June 5th 2022.

Nortura, 2019. Temahefte utmarksbeite til sau.

Norwegian Ministry of Agriculture and Food, 2004. FOR-2004-04-22-665. Regulation on keeping cattle (in Norwegian). https://lovdata.no/dokument/SF/forskrift/2004-04-22-665, accessed March 1st, 2020.

Norwegian Ministry of Agriculture and Food, 2015. FOR-2015-06-18-761 Regulation on the use of animals in experiments (in Norwegian). https://lovdata.no/dokument/SF/forskrift/2015-06-18-761?q=forskrift%20om%20bruk%20av%20dyr, Accessed August 1st, 2022.

Norwegian Ministry of Agriculture and Food., Forskrift om velferd hos småfe, [Regulation on small ruminant welfare]https://lovdata.no/dokument/SF/forskrift/2005-02-18-160. Accessed April 20th, 2020.

Oh, S.-I., Kim, J.W., Kim, J., So, B., Kim, B., Kim, H.-Y., 2020. Molecular subtyping and antimicrobial susceptibility of Streptococcus dysgalactiae subspecies equisimilis isolates from clinically diseased pigs. J. Vet. Sci. 21.

Olde Riekerink, R., Barkema, H., Kelton, D., Scholl, D., 2008. Incidence rate of clinical mastitis on Canadian dairy farms. J. Dairy Sci. 91, 1366-1377.

Oppegaard, O., Mylvaganam, H., Skrede, S., Kittang, B.R., 2018. Exploring the arthritogenicity of Streptococcus dysgalactiae subspecies equisimilis. BMC Microbiol. 18, 1-10.

Oppegaard, O., Mylvaganam, H., Skrede, S., Lindemann, P.C., Kittang, B.R., 2017. Emergence of a Streptococcus dysgalactiae subspecies equisimilis stG62647-lineage associated with severe clinical manifestations. Sci. Rep. 7, 1-10.

Park, M.J., Eun, I.-S., Jung, C.-Y., Ko, Y.-C., Kim, Y.-J., Kim, C.-k., Kang, E.-J., 2012. Streptococcus dysgalactiae subspecies dysgalactiae infection after total knee arthroplasty: a case report. Knee Surgery & Related Research 24, 120.

Persson Waller, K., Bengtsson, B., Lindberg, A., Nyman, A., Unnerstad, H.E., 2009. Incidence of mastitis and bacterial findings at clinical mastitis in Swedish primiparous cows—Influence of breed and stage of lactation. Vet. Microbiol. 134, 89-94.

Pightling, A.W., Pettengill, J.B., Luo, Y., Baugher, J.D., Rand, H., Strain, E., 2018. Interpreting whole-genome sequence analyses of foodborne bacteria for regulatory applications and outbreak investigations. Front. Microbiol. 9, 1482.

Pinho, M.D., Erol, E., Ribeiro-Gonçalves, B., Mendes, C.I., Carriço, J.A., Matos, S.C., Preziuso, S., Luebke-Becker, A., Wieler, L.H., Melo-Cristino, J., 2016. Beta-hemolytic Streptococcus dysgalactiae strains isolated from horses are a genetically distinct population within the Streptococcus dysgalactiae taxon. Sci. Rep. 6, 1-9.

Rato, M.G., Nerlich, A., Bergmann, R., Bexiga, R., Nunes, S.F., Vilela, C.L., Santos-Sanches, I., Chhatwal, G.S., 2011. Virulence gene pool detected in bovine group C Streptococcus dysgalactiae subsp. dysgalactiae isolates by use of a group A S. pyogenes virulence microarray. J. Clin. Microbiol. 49, 2470-2479.

Regulation, E.P.a.C.o.E.U., 2016. (EU) 2016/679. https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32016R0679&from=EN. Accessed October 1st, 2020.

Ruegg, P.L., 2017. A 100-Year Review: Mastitis detection, management, and prevention. J. Dairy Sci. 100, 10381-10397.

Rutherford, S.-J., Jeckel, S., Ridler, A., 2015. Characteristics of sheep flocks affected by Streptococcus dysgalactiae arthritis. Vet. Rec. 176, 435-435.

Rutherford, S.J., Rycroft, A.N., Ridler, A.L., 2014. Sources of Streptococcus dysgalactiae in English and Welsh sheep flocks affected by infectious arthritis (joint ill). Vet. Rec. 174, 579.

Ryan, D., Rothwell, J., Hornitzky, M., 1991. Streptococcus dysgalactiae infection in calves. Aust. Vet. J. 68, 210-210.

Schuchat, A., 1999. Group B streptococcus. The Lancet 353, 51-56.

Schukken, Y., Grommers, F., Van de Geer, D., Erb, H., Brand, A., 1990. Risk factors for clinical mastitis in herds with a low bulk milk somatic cell count. 1. Data and risk factors for all cases. J. Dairy Sci. 73, 3463-3471.

Schwan, O., Holmberg, O., 1979. Heifer mastitis and dry-cow mastitis: A bacteriological survey in Sweden. Vet. Microbiol. 3, 213-226.

Segerman, B., 2020. The most frequently used sequencing technologies and assembly methods in different time segments of the bacterial surveillance and RefSeq genome databases. Frontiers in Cellular and Infection Microbiology 10, 527102.

Sensini, A., Tissi, L., Verducci, N., Orofino, M., von Hunolstein, C., Brunelli, B., Malà, G.L., Perocchi, F., Brunelli, R., Lauro, V., 1997. Carriage of group B Streptococcus in pregnant women and newborns: a 2-year study at Perugia General Hospital. Clin. Microbiol. Infect. 3, 324-328.

Smith, K.L., Hogan, J.S., 1993. Environmental mastitis. Vet. Clin. North Am. Food Anim. Pract. 9, 489-498.

Spratt, B.G., Maiden, M.C., 1999. Bacterial population genetics, evolution and epidemiology. Philos. Trans. R. Soc. Lond. B Biol. Sci. 354, 701-710.

Statens Legemiddelverk., 2012. Terapianbefaling for bruk av antibiotika på produksjonsdyr. https://legemiddelverket.no/Documents/Veterin%C3%A6rmedisin/Terapianbefalinger/Terapianbefaling_bruk%20av%20antibakterielt%20midler%20til%20produks.pdf. Accessed August 1st, 2022

Statistics Norway, 2022. Statistics for Agriculture, Forestry, Hunting and Fishing. .

Sting, R., Schaufuss, P., Blobel, H., 1990. Isolation and characterization of hyaluronidases from Streptococcus dysgalactiae, S. zooepidemicus and S. equi. Zentralblatt für Bakteriologie 272, 276-282.

Swinson, V., 2021. An update on joint ill in sheep. Vet. Rec. 188, 24-26.

Taponen, S., Liski, E., Heikkilä, A.-M., Pyörälä, S., 2017. Factors associated with intramammary infection in dairy cows caused by coagulase-negative staphylococci, Staphylococcus aureus, Streptococcus uberis, Streptococcus dysgalactiae, Corynebacterium bovis, or Escherichia coli. J. Dairy Sci. 100, 493-503.

TINE, 2020. Årsrapport Kukontrollen og Geitekontrollen, Results from the Norwegian Herd Recording System and Norwegian Goat Recording System. https://medlem.tine.no/fag-og-forskning/statistikk-2020-for-kukontrollen-og-geitekontrollen/_/attachment/inline/59a4610f-2d7e-4b6f-a37a-7f43c9977306:53e8e6153c6fa6348276b5cc0bc8e9b3a58d6079/Statistikksamling%20husdyrkontrollen%202020.pdf Accessed August 1st, 2021.

Todhunter, D., Smith, K., Hogan, J., 1995. Environmental streptococcal intramammary infections of the bovine mammary gland. J. Dairy Sci. 78, 2366-2374.

Tollersrud, T.S., Tømmerberg, V., Kampen, A., Jørgensen, H.J., Gilhuus,M., 2018. Mastitt hos søye (in Norwegian). https://www.animalia.no/no/Dyr/sauehelsenett/arstid/lamming/jurbetennelse-hos-soye--en-undersokelse-i-2018/, accessed August 1st 2022.

Tømmerberg, V., Tollersrud, T., Salzano, S., Krogh, A.K., Hansen, B., Mikkelsen, L., 2017. Utbrudd av leddbetennelse hos lam [Outbreak of infectious arthritis in lambs], in Norwegian. Praksisnytt 2, 19-25.

Vakkamäki, J., Taponen, S., Heikkilä, A.-M., Pyörälä, S., 2017. Bacteriological etiology and treatment of mastitis in Finnish dairy herds. Acta Vet. Scand. 59, 1-9.

Van Seventer, J.M., Hochberg, N.S., 2017. Principles of infectious diseases: transmission, diagnosis, prevention, and control. International encyclopedia of public health, 22.

Vandamme, P., Pot, B., Falsen, E., Kersters, K., Devriese, L.A., 1996. Taxonomic Study of Lancefield Streptococcal Groups C, G, and L (Streptococcus dysgalactiae) and Proposal of S. dysgalactiae subsp. equisimilis subsp. nov. Int. J. Syst. Evol. Microbiol. 46, 774-781.

Vela, A.I., Falsen, E., Simarro, I., Rollan, E., Collins, M.D., Domínguez, L., Fernandez-Garayzabal, J.F., 2006. Neonatal mortality in puppies due to bacteremia by Streptococcus dysgalactiae subsp. dysgalactiae. J. Clin. Microbiol. 44, 666-668.

Vos, P., Garrity, G., Jones, D., Krieg, N.R., Ludwig, W., Rainey, F.A., Schleifer, K.-H., Whitman, W.B., eds., 2011. Bergey's manual of systematic bacteriology: Volume 3: The Firmicutes, Vol 3. Springer Science & Business Media.

Waage, S., Ødegaard, S., Lund, A., Brattgjerd, S., Røthe, T., 2001. Case-control study of risk factors for clinical mastitis in postpartum dairy heifers. J. Dairy Sci. 84, 392-399.

Watkins, G., 2007. Arthritis, in Diseases of Sheep, 4th ed. Edition. Blackwell Science, Oxford.

Watkins, G., Sharp, M., 1998. Bacteria isolated from arthritic and omphalatic lesions in lambs in England and Wales. The Veterinary Journal 156, 235-238.

Watt, B., Refshauge, G., 2010. An outbreak of *Streptococcus dysgalactiae* arthritis in neonatal lambs. Proceedings of the 3rd AVA/NZVA Pan Pacific Veterinary Conference.

Wente, N., Krömker, V., 2020. Streptococcus dysgalactiae—Contagious or Environmental? Animals 10, 2185.

Whist, A., Østerås, O., Sølverød, L., 2007a. Streptococcus dysgalactiae isolates at calving and lactation performance within the same lactation. J. Dairy Sci. 90, 766-778.

Whist, A.C., Østerås, O., Sølverød, L., 2007b. Staphylococcus aureus and Streptococcus dysgalactiae in Norwegian herds after introduction of selective dry cow therapy and teat dipping. J. Dairy Res. 74, 1-8.

Wißmann, J.E., Kirchhoff, L., Brüggemann, Y., Todt, D., Steinmann, J., Steinmann, E., 2021. Persistence of pathogens on inanimate surfaces: a narrative review. Microorganisms 9, 343.

Zadoks, R.N., Middleton, J.R., McDougall, S., Katholm, J., Schukken, Y.H., 2011. Molecular epidemiology of mastitis pathogens of dairy cattle and comparative relevance to humans. J. Mammary Gland Biol. Neoplasia 16, 357-372.

Zadoks, R.N., Schukken, Y.H., 2006. Use of molecular epidemiology in veterinary practice. Veterinary Clinics: Food Animal Practice 22, 229-261.

Zadoks, R.N., Tikofsky, L.L., Boor, K.J., 2005. Ribotyping of Streptococcus uberis from a dairy's environment, bovine feces and milk. Vet. Microbiol. 109, 257-265.

12 Papers I-IV

Smistad *et al. Acta Vet Scand* (2020) 62:64
https://doi.org/10.1186/s13028-020-00561-z

Acta Veterinaria Scandinavica

RESEARCH

Open Access

Flock-level risk factors for outbreaks of infectious arthritis in lambs, Norway 2018

Marit Smistad[1,2*], Cecilia Wolff[1], Tore Tollersrud[3], Vibeke Tømmerberg[3], Clare Phythian[4], Annette Hegermann Kampen[1] and Hannah Joan Jørgensen[1*]

Abstract

Background: Outbreaks of infectious arthritis in young lambs associated with *Streptococcus dysgalactiae* subspecies *dysgalactiae* (SDSD) lead to reduced animal welfare, increased use of antibiotics and economic losses for sheep farmers. Understanding risk factors is essential when developing strategies to prevent such outbreaks. This questionnaire-based cross-sectional study classified sheep flocks of respondents as cases or controls. Flock-level risk factors for outbreaks of infectious arthritis were assessed using a multivariable logistic regression model.

Results: Eighty-four of 1498 respondents (5.6%) experienced an outbreak of infectious arthritis in their flock in 2018, the year of study. Factors associated with a higher risk of outbreak were larger flock size (OR 1.3, 95% CI 1.1–1.4, per 100 lambs), plastic mesh flooring in the lambing pen (OR 3.0, 95% CI 1.7–5.3) and a lambing percentage greater than 200 (OR 2.0, 95% CI 1.1–3.5). Flocks where farmers observed infections around the ear tags of lambs also had an increased risk of outbreak (OR 2.6, 95% CI 1.6–4.3).

Conclusions: The risk factors identified in this study are characteristic of modern and intensively managed sheep farms in Norway. A distinguishing feature of Norwegian sheep farming is winter housing and indoor lambing. One might expect that this in itself is a risk factor because of high stocking densities during lambing. However, outbreaks of infectious arthritis in young lambs are reported by the industry to be a more recent phenomenon. The current study indicates that intensification of indoor management systems with larger flocks and higher production per ewe may predispose to outbreaks. The results provide a basis for further studies on transmission dynamics of SDSD in sheep flocks with indoor lambing.

Keywords: Arthritis, Joint ill, Management, Ovine, Questionnaire, SDSD, *Streptococcus dysgalactiae* subspecies *dysgalactiae*

Background

Over the past 10 years, outbreaks of infectious arthritis (joint ill) in young lambs have been a growing concern for the Norwegian sheep industry. In some farms, up to 40% of the lambs have been affected shortly after birth [1]. Not only does this present a serious animal welfare issue, but the scale and nature of antibiotic use in affected flocks is contrary to the Norwegian policies on antimicrobial use for livestock [2].

Although joint-swelling and lameness of non-infectious origin may occur in sheep, a sudden onset and rapid within-flock spread of disease in young lambs, is characteristic of infectious arthritis [3]. The disease can have several bacterial causes with *Erysipelothrix rhusiopathiae*, *Staphylococcus aureus* and *Streptococcus dysgalactiae* subspecies *dysgalactiae* (SDSD) as the major species. Some authors consider *E. rhusiopathiae* the most common cause of arthritis in lambs [4]. This infection is occasionally seen in Norway, also in lambs below

*Correspondence: marit.smistad@tine.no; hannah.jorgensen@vetinst.no
[1] Norwegian Veterinary Institute, Ullevålsveien 68, 0454 Oslo, Norway
[2] TINE SA, Postboks 7, 0901 Oslo, Norway
Full list of author information is available at the end of the article

1 month of age [5], but it typically presents in lambs between 2 and 6-months of age [3, 4]. *S. aureus* is also a relatively common cause of arthritis in lambs, but outbreaks are often a complication of tick-associated pyemia in lambs on pasture [4].

SDSD has been identified as the most important cause of outbreaks of infectious arthritis in young lambs in England and Wales [3, 6], and as a cause of outbreaks of polyarthritis in lambs in Australia [7], New Zealand [8] and Spain [9]. Typically, SDSD arthritis affects lambs under 4 weeks of age [10], and clinical features include acute lameness, fever and recumbency. The lambs are often dull and unthrifty, and some show signs of secondary pneumonia or meningitis [3, 10].

In order to document the microbiological causes of outbreaks of infectious arthritis in young lambs in Norway, the Norwegian Sheep Health Service and veterinary practitioners conducted a pilot study during the lambing seasons of 2016, 2017 and 2018. They visited 19 flocks, 12 of which experienced an outbreak at least one of the seasons. Approximately two thirds of the clinical cases occurred within the first week of life [1]. Upon bacteriological culturing, SDSD was identified from joint aspirates in 11 of the 12 flocks (Tømmerberg et al., unpublished data), indicating that SDSD is the main causative agent of outbreaks of infectious arthritis in young lambs in Norway.

Previous studies on SDSD have suggested unhygienic conditions in the lambing pen [3, 11], high stocking densities [10] and early ear tagging to be associated with an increased risk of outbreaks of infectious arthritis in lambs [12, 13]. Contaminated stomach tubes [14] and inadequate hygiene when providing lambing assistance [15] have been proposed as possible routes of bacterial transmission between animals.

In Norway, anecdotal reports from the sheep industry suggest that outbreaks of SDSD joint ill are mainly associated with large, intensively managed sheep flocks with many hundreds of lambs born indoors within a few weeks [1]. Winter-housed sheep are fed high quality silage and concentrate, and a lambing percentage of 250 is common in these flocks. Stocking densities are high, and the indoor environment can be unfavourable at the peak of the lambing season. However, well managed flocks with good hygiene have also experienced outbreaks [1].

The Norwegian sheep industry wishes to develop evidence-based management strategies to prevent outbreaks of infectious arthritis in sheep flocks with indoor laming. The objective of the study, therefore, was to perform a survey to identify flock-level risk factors for outbreaks of infectious arthritis in lambs under Norwegian management conditions.

Methods

Structure of the Norwegian sheep industry

In 2018, there were approximately 14,000 sheep farms in Norway [16], and 40% of these were members of the Norwegian Sheep Recording System (NSRS) [17]. The average flock size among NSRS-members was 86 winter-housed sheep, and 6% of the flocks have more than 200 ewes [17]. The animals are kept for meat and wool, and the main breed is Norwegian White Sheep, a composite crossbreed accounting for about 70% of the national population. Typically, flocks are housed during the winter season from mating until 1–2 weeks after lambing. Lambing starts between March and May depending on the local climate. The sheep and their lambs are let out onto spring pasture for a few weeks, before they are sent to summer pasture in outfield grazing areas in the woods or mountains or kept on lowland grazing areas. The lambs are slaughtered in the period between August and November, and the average carcass weight is 18.4 kg [18]. In Norway, it is mandatory to ear-tag lambs within 30 days after birth, and most sheep farmers tag their lambs the first week of life. Tail docking and castration is prohibited according to the animal welfare legislation [19].

Study design and data collection

This cross-sectional study of the lambing season of 2018 was based on a survey. On the 14th of March 2019, the Norwegian Sheep Health Services distributed the online questionnaire to all members of the NSRS with a registered e-mail address (n = 5374). The questionnaire was also made available by link on the web page of the Norwegian Meat and Poultry Research Centre (www.Animalia.no). The survey closed on the 25th May 2019, after one email reminder.

Questionnaire

The questionnaire, created in Questback (www.Questback.com), included 86 questions (Additional file 1), and took about 20 min to complete. Most questions were closed, or semi-closed, and where relevant, space was provided for comments. Before distribution, three sheep farmers, with no history of infectious arthritis outbreaks in their flocks, tested the questionnaire to ensure clarity.

To provide data on suspected risk factors, the 86 questions covered: (i) flock characteristics (ii) housing (iii) feeding routines (iv) management practices in general and during lambing for the season of 2018, and (v) the occurrence of infectious arthritis during the previous four lambing seasons (2015–2018). Data were collected and stored according to the General Data Protection Regulation (GDPR) [20]. Farmers (respondents) could

choose to answer the questionnaire anonymously by providing their postal code instead of their farmer-id.

Definition of case and control flocks

Survey data from flocks with more than 40 lambs born during the lambing season of 2018 were reviewed for inclusion as cases or controls. The following inclusion criteria for case-flocks were used: (i) the farmer reported that five percent or more of the lambs were affected with arthritis in the lambing season of 2018, (ii) the affected lambs were 4 weeks or younger and (iii) the clinical signs of affected lambs were lameness in combination with either joint swelling, pyrexia, dullness or respiratory signs. Farmers that reported more than five percent of lambs affected with arthritis but described lameness and interdigital swelling as the only clinical signs, were excluded from the analyses because those symptoms are more likely to be associated with interdigital abscesses than arthritis. Survey data from remaining respondents were included as control-flocks.

Data management

Raw data were exported and stored in Excel (Microsoft Corp, Redmond, WA, USA, 2016) and analysed with Stata (Release 14.2, Stata Corp LLC, USA, 2015). Variable categories with five or fewer observations were amalgamated when biologically or logically possible, or not included the multivariable model. When feasible, multiple questions within the same topic were combined into one variable, e.g. the variable "environment in the shed" was created from four statements in the questionnaire regarding the environment.

For flocks with missing data on flock size, lamb mortality and breed, the information was electronically retrieved from the NSRS, when available. Variables with more than 10% missing observations were not considered for multivariable analysis. Respondents with more than 15% missing variables were excluded from the analyses.

Data analysis

Before analysis, the hypothetical relationship between the outcome and exposures was outlined in a causal diagram, based on literature review, biological knowledge and clinical observations of the research team. Categorical variables were described by cross tabulation against the outcome. Continuous variables were plotted against the outcome variable using boxplots. Linearity was assessed by plotting continuous variables against the logit transformed outcome with Lowess smoothing plots [21].

First, unconditional associations between the dependent variable and each of the potential risk factors were screened using Chi^2-tests (categorical variables). Flock size was rescaled by dividing it by 100 to aid interpretation of the OR. Variables with a P-value ≤ 0.2 were tested in the multivariable analysis. Spearman rank correlations (categorical ordinal), tabulation (categorical nominal) and Pearson correlations (continuous) were used to assess collinearity between the predictors [21]. If two variables showed collinearity ($r > 0.7$, where applicable) the one with the lowest P-value or suspected highest biological relevance was kept for further analysis.

A multivariable logistic regression model was used to evaluate the risk factors for being a case flock. The model was built using manual backwards elimination, with the logit function. Variables were removed from the model based on likelihood ratio-test at each step, with $P < 0.05$ as a criterion for retention [21].

To assess confounding, variables excluded during the reduction were re-entered one at a time when all remaining variables were significant. A variable was considered a confounder if there was a greater than 20% change in any coefficients' estimates when the variable was included. Biologically plausible interaction terms between main effects were tested in the model. The fit of the model was evaluated with Hosmer–Lemeshow goodness of fit test with the data partitioned into 10 deciles. Outliers and influential observations were identified by examinations of observations with Pearson residuals > 2 or < -2, deviance residuals > 2 or < -2 or with leverage (hat) $> 3 *$ mean hat.

The representativeness of the sheep flocks of respondents was examined by comparing the breed composition, the mean flock size and the mean lamb mortality with averages reported by the NSRS [17]. The geographical distribution of respondent flocks per county was visually compared with the distribution of all sheep flocks in Norway. Flock size and lamb mortality percentage of the flocks in the data set used in the multivariable analysis were compared to the complete dataset including all respondents to assess differences that could potentially bias the results.

Results

Study population

A total of 1761 farmers responded to the questionnaire. Of these, 1490 responded to the e-mail, giving a response rate of 27%. In addition, 271 farmers responded via the link on the web page. Data from respondents were excluded from the analyses if the flock had less than 40 ear-tagged lambs ($n = 210$), answers had more than 15% missing values ($n = 33$), were duplicates ($n = 12$), or the farmer reported that more than five percent of the lambs were affected but described lameness and interdigital swelling as the only clinical signs indicating a problem of interdigital abscesses rather than arthritis ($n = 8$). Among the 1498 flocks that met the inclusion criteria and were

available for descriptive statistics, 84 (5.6%) were classified as case flocks. The final dataset, without missing values, used for the multivariable analyses included 77 case flocks and 1178 control flocks.

Flock characteristics and management practices

The number of ear-tagged lambs was used as an indicator of flock size. The flock size ranged from 40 to 1323 ear-tagged lambs. The median flock size was 226 ear-tagged lambs [interquartile range (iqr) 133–371] in case flocks and 134 (iqr 84–226) in control flocks. There was an association between being a case flock and larger flock size in the univariable analysis ($P < 0.001$). The case flocks had a median overall lamb mortality of 2.5% (iqr 1.6–4.3) while the control flocks had a median overall lamb mortality of 1.9% (iqr 0.9–3.3).

Thirty-three of the 44 explanatory variables tested in the univariable analysis are presented in Tables 1, 2, 3, 4, and 5. Any association between outbreaks and the presence of other animal species on the farm (10 variables) were tested in the univariable analysis, but not in the multivariable model due to more than 15% missing values. A question about the total indoor area in the shed during the winter season was part of the questionnaire (not shown), but could not be used because many respondents commented that they use additional areas during lambing or had difficulty in defining the relevant areas. Many also left the question blank.

The attack rate of infectious arthritis among the case flocks was 5–10% in 69 flocks (82%), 11–20% in 12 flocks (14%), while three farmers (4%) reported that 21% or more of the lambs were affected. Lameness or swollen joints were reported as clinical signs in all the case flocks. In addition, "general apathy" was reported as a clinical sign in 25% of the flocks, and recumbency or reluctance to move was reported in 13% of the flocks. Only 6%, 3% and 1% reported navel infection, dyspnea and coughing, respectively.

Of the case flocks, 66 (79%) had experienced an outbreak of infectious arthritis in at least one of the lambing seasons before 2018 (Table 1). Unconditional logistic regression on the factor "previous outbreak" gave an OR of 29 (95% CI 16.5–50.6), but as this variable was considered an intervening factor it was not included in the multivariable model. Among the farmers that had an outbreak of arthritis in young lambs before 2018 ($n = 212$), 92% had introduced measures to prevent future outbreaks (Fig. 1). Disinfection of navels was the most commonly reported measure (55%).

In flocks with outbreaks in 2018 or one of the three previous seasons, the diagnosis of infectious arthritis was made by a veterinarian for 48% of the flocks, and by the farmer him-/herself for 46% of the flocks. Four percent had submitted samples for bacteriological culture from affected lambs, and 2% had submitted dead lambs for necropsy.

Table 1 Description of flock data variables tested in univariable screening (Chi^2-test)

Variable	Categories	Total		Case flocks (n = 84)		Control flocks (n = 1414)		
		n	%	n	%	n	%	P-value
Number of lambs (ear-tagged, categorized)[a]	< 200	1014	67.7	36	42.9	978	69.2	< 0.0001
	200–500	427	28.5	36	42.9	391	27.7	
	> 500	57	3.8	12	14.3	45	3.2	
Outbreak of infectious arthritis before 2018[c]	No	1286	85.9	18	21.4	1268	89.7	< 0.0001
	Yes	212	14.2	66	78.6	146	10.3	
Lambing percentage[b]	≤ 200	592	39.7	19	22.6	573	39.7	0.001
	> 200	898	60.3	65	77.4	833	60.3	
Breed[b]	Breed other than Norwegian White Sheep	301	20.5	7	8.9	294	21.1	0.009
	Norwegian White Sheep	1171	79.6	72	91.1	1099	78.9	
Start of lambing season[b]	April	1181	79.2	62	73.8	1119	73.8	0.038
	May	189	12.7	9	10.7	180	12.7	
	March	121	8.1	13	15.5	108	7.7	
Length of lambing season[b]	< 4 weeks	921	61.5	44	52.4	877	62.1	0.076
	> 4 weeks	576	38.5	40	47.6	536	37.9	

[a] Not tested in the multivariable model due to collinearity with number of ear-tagged lambs (continuous, not shown)

[b] Tested in multivariable model

[c] Intervening variable, not tested in the multivariable model

Table 2 Description of variables for housing conditions tested in the univariable screening (Chi²-test)

Variable	Categories	Total		Case flocks (n = 84)		Control flocks (n = 1414)		
		n	%	n	%	n	%	P-value
Flooring type in lambing pen[a]	Metal mesh flooring	649	43.4	26	31.0	623	44.1	< 0.0001
	Plastic mesh flooring	314	21.0	41	48.8	273	19.3	
	Other/combinations	533	35.6	17	20.2	516	36.6	
Flooring type for lambs before let out onto pasture[b]	Metal mesh flooring	348	23.2	21	25.3	327	23.2	< 0.0001
	Plastic mesh flooring	145	9.7	26	31.3	119	8.4	
	Straw bed/deep litter	310	20.8	10	12.1	300	21.3	
	Other/combinations	691	46.3	26	31.3	665	47.1	
Bedding material in single pens[c]	Not using bedding material	355	23.4	20	24.1	335	23.8	0.005
	Straw	493	33.1	42	50.6	451	32.0	
	Sawdust	178	12.0	9	10.8	169	12.0	
	Hay	167	11.2	6	7.2	161	11.4	
	Other bedding materials or combinations	271	18.2	6	7.2	265	18.8	
	Not using single pens	27	1.8	0	0	27	1.9	
Age of the shed[a]	> 10 years	952	63.9	41	48.8	911	64.8	0.01
	Rebuilt/modernized the last 10 years	282	18.9	21	25.0	261	18.6	
	< 10 years	256	17.2	22	26.2	234	16.6	
Environment in the shed after lambing vs. before[a]	Dry	611	42.4	26	31.0	585	43.1	0.021
	More humid	426	29.6	24	28.6	402	29.7	
	More humid and dirtier	403	28.0	34	40.5	369	27.2	
Time spent in single pens after lambing[c]	≥ 3 days	840	56.2	38	45.2	802	56.8	0.031
	1–2 days	628	42.0	46	54.8	582	41.3	
	Not using single pens	27	1.8	0	0.0	27	1.9	
Group size (ewes before lambing)[a]	≤ 15	767	53.0	36	42.9	731	53.7	0.086
	16–30	441	30.5	28	33.3	413	30.3	
	> 30	238	16.5	20	23.8	218	16.0	
Housing type	Uninsulated	405	27.3	19	22.6	386	27.6	0.512
	Insulated	745	50.2	47	56.0	698	49.9	
	Other housing type, outdoors combination	333	22.5	18	21.4	315	22.5	

[a] Tested in multivariable model

[b] Intervening variable, not tested in the multivariable model

[c] Not tested in the multivariable model due to categories with five or fewer observations

Antibiotic treatment was reported as administered to most of the affected lambs in 74 (89%) of the case flocks, and a few animals in seven (8%) case flocks. Respondents of 2 (2%) case flocks reported that no affected lambs had been treated with antibiotics. The most common route of administration of antibiotics was injection (n = 74, 88%), but affected lambs were treated *per os* in five case flocks (6%). The reported duration of treatment was 2–3 days (42%), 4–5 days (39%) or more than 5 days (16%).

In larger flocks, early ear tagging was more common. Among flocks with < 200 lambs (n = 1012); 200–500 lambs (n = 426); and > 500 lambs (n = 57), 41%, 66% and 91% of the respondents, respectively, reported to perform ear tagging within 24 h after birth.

Multivariable analysis

Altogether 44 variables were screened in the univariable analysis, and 20 were offered to the multivariable model (Tables 1, 2, 3, 4, and 5). Risk factors that remained in the final model are shown in Table 6. The overall likelihood ratio Chi² test (5 df) P-value of the model was < 0.001.

None of the removed variables had a confounding effect on any variable parameter estimate in the final model. The interaction terms flock size * flooring and flock size * lambing percentage were non-significant (P > 0.05). The model showed acceptable fit according to the Hosmer–Lemeshow goodness of fit test, with chi squared (df 8) = 10.1 (P = 0.26). Examining the observations with Pearson residuals > 2 (n = 54), deviance > 2

Table 3 Description of variables for management at lambing tested in the univariable screening (Chi²-test)

Variable	Categories	Total		Case flocks (n = 84)		Control flocks (n = 1414)		
		n	%	n	%	n	%	P-value
Observed infections around ear tag[a]	No (never/rarely)	1103	73.7	44	52.4	1059	75.0	< 0.0001
	Yes (sometimes, often)	393	26.3	40	47.6	353	25.0	
Routines for colostrum supply[b]	Observe that they suck, not using stomach tubes	504	33.7	11	13.1	493	34.9	< 0.0001
	Observe that they suck, use stomach tubes routinely/when needed	931	62.2	67	79.8	864	61.1	
	Not consistent, no clear routines	62	4.1	6	7.1	56	4.0	
How often are stomach tubes used for colostrum supply[a, b]	Not using stomach tubes	550	36.8	15	17.9	535	37.9	< 0.0001
	Sometimes (1–10% of the lambs)	776	51.9	49	58.3	727	51.5	
	Relatively often (> 10% of the lambs)	170	11.4	20	23.8	150	10.6	
Disinfection of navels[a]	Never/rarely, sometimes	633	42.3	22	26.2	611	43.2	0.002
	Yes	864	57.7	62	73.8	602	56.8	
Age at ear tagging[a]	1 day	746	49.9	47	56.0	699	49.5	0.011
	2 days	370	24.8	27	32.1	343	24.3	
	≥ 3 days	379	25.4	10	11.9	369	26.2	
Statement: as far as possible the ewe and her lambs are left in peace during and immediately after lambing[a]	Fully agree	954	64.1	43	51.1	911	64.8	0.017
	Partly agree/disagree	535	35.9	40	48.2	495	35.2	
% of ewes needing assistance during lambing[a]	0–10%	443	30.2	16	19.3	427	30.9	0.031
	11–20%	430	29.3	23	27.7	407	29.4	
	> 20%	594	40.5	44	53.0	550	39.7	
Use of disinfectant on ear tag	No/sometimes	908	61.1	54	65.9	854	60.8	0.36
	Yes	578	38.9	28	34.2	550	39.2	

[a] Tested in multivariable model

[b] Not tested in the multivariable model due to collinearity with another variable (with the same letter)

(n = 43) or leverage > 3 * mean hat (n = 43) did not show any patterns with regard to values of explanatory variables. Refitting the model without different combinations of these observations had a small effect on OR estimates, and goodness of fit was still acceptable.

The geographical distribution of flocks corresponded well with the distribution of flocks in Norway (data not shown). Flock characteristics used to assess the representativeness of the respondents are presented in Table 7.

The median flock size (ear tagged lambs) was 138 in flocks with more than 40 lambs (n = 1498), and 141 in the dataset that was used in the multivariable analysis (n = 1178). The median overall lamb mortality was three percent in both groups.

Discussion

This study confirms that outbreaks of infectious arthritis in lambs present an important animal health issue in Norway that needs to be managed to reduce the negative impacts on animal welfare, antibiotic usage, and profitability. Identification of flock-level risk factors is fundamental to development of evidence-based management strategies to prevent future outbreaks.

In this study, almost 6% of the included sheep flocks had experienced an outbreak of infectious arthritis in young lambs in 2018, fitting the characteristics of SDSD outbreaks. Assuming the respondents were representative for Norwegian sheep farmers with more than 40 lambs, and that 2018 was a representative year, the study suggests that 6% of Norwegian sheep flocks may be affected by an outbreak during the lambing season. This could be an overestimation of the true flock level prevalence, as the farmers that have experienced outbreaks are probably more likely to respond to the questionnaire. Without historical data it is not possible to evaluate whether this represents an increase compared to previous years or not.

Following pilot studies, the sheep health services suggested that outbreaks of arthritis are associated with large, intensively managed flocks [1]. This is supported in the present study, which confirms that flock size and a lambing percentage greater than 200 percent are risk factors for an outbreak. Farmers with

Table 4 Description of variables for hygienic measures tested in the univariable screening (Chi²-test)

Variable	Categories	Total		Case flocks (n = 84)		Control flocks (n = 1414)		
		n	%	n	%	n	%	P-value
Hand hygiene: statement: "I always wash my hands and/or change gloves after handling diseased animals"[a]	Fully agree	1309	87.4	66	78.6	1243	88.0	0.012
	Partly agree/disagree	188	12.6	18	21.4	170	12.0	
Hand hygiene when performing lambing assistance[a]	Adequate (always hand wash or change of gloves)	1423	95.3	76	90.5	1347	95.6	0.03
	Inadequate (not consistent hand hygiene measures)	70	4.7	8	9.5	62	4.4	
Is the bedding material in the single pens changed between lambings?[b]	Usually/always	747	66.2	47	77.1	700	65.6	0.126
	Sometimes/never	354	31.4	14	23.0	340	31.9	
	Do not use single pens	27	2.4	0	0.0	27	2.53	
Are the single pens cleaned between lambings?	Always	99	29.3	7	35.0	92	28.9	0.563
	Sometimes/never	239	70.7	13	65.0	226	71.1	
How often are the bottles/stomach tubes cleaned?	Between every lamb	722	48.4	39	46.4	683	48.5	0.710
	Once daily	492	33.0	31	36.9	461	32.7	
	When needed	279	18.7	14	16.7	265	18.8	
How often is the shed cleaned (washed)?	Annually	988	66.0	57	67.9	931	65.9	0.710
	Less often than annually	509	34.0	27	32.1	482	34.1	

[a] Tested in multivariable model

[b] Not tested in the multivariable model due to categories with five or fewer observations

Table 5 Description of variables related to feeding tested in the univariable analysis (Chi²-test)

Variable	Categories	Total		Case flocks (n = 84)		Control flocks (n = 1414)		
		n	%	n	%	n	%	P-value
How often is concentrate offered?[b]	Twice daily	896	60.5	51	58.0	849	60.6	< 0.001
	Once daily	314	21.2	16	18.5	299	21.3	
	≥ Thrice daily	87	5.9	7	8.6	80	5.7	
	Automat	62	4.2	11	13.6	51	3.6	
	Not giving concentrate	123	8.3	2	1.2	122	8.7	
Ewes' faecal consistency when lambing starts[a]	Firm pellets	1234	84.4	57	71.3	1177	85.1	0.001
	Soft paste or diarrhoea	229	15.7	23	28.8	206	14.9	
kg concentrate before lambing[a]	< 0.5 kg	726	49.0	29	34.9	697	49.8	0.004
	0.5–1 kg	640	43.2	41	49.4	599	42.8	
	> 1 kg	116	7.8	13	15.7	103	7.4	
Type of forage[a]	Silage and hay	326	21.8	9	10.7	317	22.5	0.008
	Hay	211	14.1	8	9.5	203	14.4	
	Silage	958	64.1	67	79.8	891	63.2	
Kg concentrate after lambing	< 1 kg	755	51.6	40	50.0	715	51.7	0.880
	1–1.5 kg	497	34.0	27	33.8	470	34.0	
	> 1.5 kg	210	14.4	13	16.3	197	14.3	

[a] Tested in multivariable model

[b] Intervening variable, not tested in the multivariable model

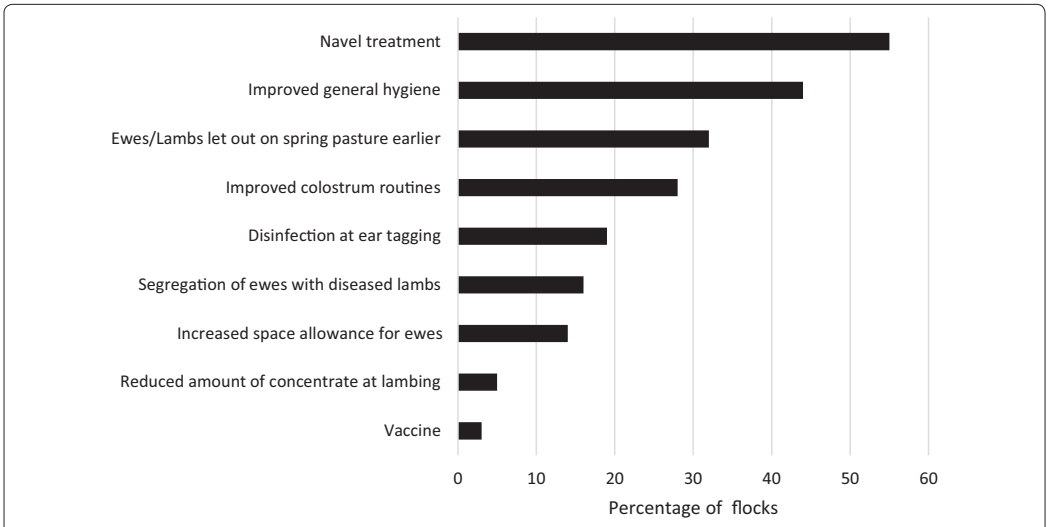


Fig. 1 Preventive measures introduced in 195 of the 212 sheep flocks that had experienced an outbreak of infectious arthritis between 2015 and 2017. Sixty-six of these flocks were case flocks in 2018

Table 6 Final multivariable logistic regression model for flock risk factors for outbreaks of infectious arthritis in lambs

Variable	Categories	n cases	n controls	OR	95% CI
Flock size[a]				1.3	1.1–1.4
Flooring in lambing pen	Metal mesh flooring	23	525	Base	
	Plastic mesh flooring	38	235	3.0	1.7–5.3
	Other	16	418	0.9	0.5–1.7
Observed infections/pus around ear tag wounds	No (never/rarely)	39	874	Base	
	Yes (sometimes, often)	38	304	2.6	1.6–4.3
Lambing percentage	≤ 200	17	468	Base	
	> 200	60	710	2.0	1.1–3.5

[a] Number of ear-tagged lambs, divided by 100

Table 7 Flock characteristics used to assess the representativeness of the respondents

Parameter	Respondents	Members of NSRS[a]
Flock size (winter-housed ewes), mean	79	86
Lamb mortality (%), mean	4.4	4.4
Breed composition (%)	74.4[b]	70[c]

[a] Norwegian Sheep Recording System

[b] Flocks with Norwegian White as main breed

[c] Percentage of ewes (members of the NSRS) that are Norwegian White

larger flocks often have several hundreds of lambs born indoors within few weeks. A higher number of susceptible animals in a confined space will increase the risk of bacterial transmission. Another variable associated with intensive management; feeding of ewes with a high concentrate level prior to lambing, was the last factor to be eliminated from the model and was not statistically significant (LR-test P-value 0.08). Feeding with a high level of concentrate was not expected to predispose to outbreaks in itself, but through its association with ruminal acidosis and loose faecal consistency it was suspected it might negatively affect hygiene in the sheep shed and predispose to infections. Future studies involving farm visits can more accurately register and evaluate feeding routines, hygiene and faecal consistence than a questionnaire-based study.

Based on survey-data it was not possible to measure stocking density for each farm. Many respondents

reported to have several sheds and to use provisional areas during lambing, leaving this variable difficult to assess. However, stocking density is expected to be relevant. Many veterinarians report to the sheep health services that over-crowding at lambing and a high turnover rate in the lambing areas are common features of flocks with outbreaks.

Not surprisingly, and in agreement with experiences of the sheep health services, flocks that have had outbreaks of infectious arthritis in previous years were at higher risk of outbreaks in the lambing season 2018. This could be associated with exposure to the same risk factors year after year, or the existence of a bacterial reservoir, presumably SDSD, in these flocks.

Anecdotal reports from some farmers and veterinary practitioners had indicated that plastic mesh flooring may be a risk factor for outbreaks. The current study confirms that flocks with lambing on plastic mesh flooring are at higher risk of arthritis outbreaks compared to flocks with lambing on other floor types. Compared to metal mesh flooring, which has been the most common floor in Norwegian sheep sheds, the plastic mesh floor has a larger surface area leaving more fluids, faeces and possibly bacterial biofilm on the surface in the lambing pen. Norwegian ewes often lamb directly onto the floor, without bedding material and are subsequently moved to individual pens with bedding. Possibly, the plastic floor contributes to transmission of SDSD to the lambs after birth.

Case reports have suggested early ear-tagging as a risk factor of infectious arthritis in lambs [12, 13]. While the univariable results in this study supported this, it was not verified in the multivariable analysis. Inflammatory reactions at the site of the ear tag, however, remained in the final model. Earlier research indicated that inflammatory reactions and infections around the ear tag are common findings following tagging of older lambs [22]. In the current study, no association was found between age at ear tagging and the occurrence of infections around the ear tag (Chi^2 test, $P = 0.58$, not shown). However, 92% of the respondents reported that they tag their lambs within the first 5 days of life.

Seventy-nine percent of respondents with case flocks reported to have had an outbreak at least one of the previous seasons, and 92% of these had introduced preventive measures. This is probably the reason why some preventive measures recommended to prevent infectious arthritis in young lambs [3, 10], such as navel disinfection, ensuring adequate colostrum intake and disinfection of the skin at ear tagging, came out as risk factors rather than protective in the univariable results. Implementation of these measures are most likely a consequence, rather than a cause, of outbreaks.

Inadequate hand hygiene when performing lambing assistance was identified as a possible risk factor for outbreaks of infectious arthritis in in the univariable analysis but was not confirmed in the multivariable model. Rutherford et al. [15], suggested lambing assistance as a possible means of transmission of the bacteria within the flock, and proposed that vaginal colonization of the ewe may be an important reservoir. The proportion of ewes needing lambing assistance can be related to the feeding in late pregnancy and possibly genetic factors. In case flocks 53% of farmers claimed to assist more than 20% of their sheep in lambing while 40% of farmers in control flocks assisted more than 20% of their sheep. There was no association between inadequate hygiene and assisting a high percentage of ewes in lambing. The farmer's attitude and level of experience probably play a role. Adherence to hygienic principles when providing lambing assistance may be an indicator of the general hygiene practice of the farmer, which could have wider implications for occurrence and transmission of infectious diseases. Hand washing and routines for changing gloves can also be related to the intensity of lambing, the availability of sufficient staff to handle many lambings in a short period of time, the barn design and the accessibility of washing facilities.

In the current study, the outcome of interest and the explanatory variables were collected using a questionnaire, and the sheep flocks were classified as cases or controls based on farmer reports. Only four and two percent of the case flock-respondents reported that they had submitted joint aspirates for culturing or lambs for necropsy, respectively. Without a bacteriological diagnosis from the flocks, we cannot rule out that some had outbreaks caused by other bacteria than SDSD. However, in light of results from the Norwegian pilot study, the age of the affected lambs (≤ 4 weeks) and the fact that outbreaks occurred during the lambing season while lambs were indoors, point to SDSD as the most likely cause.

The cut off for defining an outbreak was set at 5% of lambs affected. Clinical signs, age of diseased lambs and type of treatment were used to classify flocks correctly. The majority of farmers that reported outbreaks of infectious arthritis also described typical clinical signs. Only eight flocks were excluded because clinical signs were inconsistent with arthritis. There are likely to be differences in knowledge, routines for disease recording and accuracy of farmer recollections from the previous lambing season, and misclassifications of flocks may have occurred. In general, farmer-reported observations should be interpreted with some care, especially evaluation of their own management routines.

A questionnaire was used to reach out to as many farmers as possible, and because data on most of the

explanatory variables were not available. Moreover, the outcome of interest, cases of infectious arthritis, is usually noted on mandatory paper-based health records only, and is not reported unless the animal receives veterinary treatment. In future, flock visits to observe housing and management, and to perform clinical examinations and sampling of affected animals for bacteriological culturing would be recommended.

Only farmers that were members of the Norwegian Sheep Recording System (NSRS) received the questionnaire, hence results may not be generalizable to non-member flocks. The representativeness of the NSRS data in comparison to the Norwegian sheep population has not been evaluated in detail, except on selected production parameters where differences were small [17]. Given that membership represents 40 percent of all sheep flocks in Norway, and knowledge generated from the long history of excellent census and mandatory health and medicines data for sheep and other livestock in Norway, the participating farms are considered representative of the management systems and flock sizes found across Norway.

There was no previous knowledge on the prevalence of infectious arthritis in lambs in Norwegian sheep flocks, and instead of carrying out a sample calculation and a subsequent sampling of herds, all members of the NSRS were invited to participate in the survey. Using the rule of thumb that a dataset with a rare outcome should contain at least 10*(number of predictors in the model + 1) positive outcomes [21], our data with 77 case flocks would allow a model to be fitted with approximately 7 predictors, which is more than in the final model. Nevertheless, it is possible that additional management factors would have been included in the final model if the number of case flocks, and the statistical power, had been higher.

With the knowledge gained in this first study on risk factors for outbreaks of infectious arthritis in a Norwegian setting, further studies can be targeted towards the risk factors identified here as well as other interesting parameters that were not included in the final model.

Conclusions

In this study, 5.6% of the sheep flocks had an outbreak of infectious arthritis in young lambs. Flocks that had suffered a previous outbreak were at higher risk of an outbreak in the lambing season 2018. The risk of outbreak increased with larger flock size, and in flocks with a lambing percentage greater than 200. In addition, lambing on plastic mesh flooring and infection or inflammation at the site of ear tags of lambs were associated with the risk of outbreak.

The risk factors identified in this study are characteristic of modern and intensively managed Norwegian sheep flocks. A distinguishing feature of Norwegian sheep farming is winter housing and indoor lambing.

An important task of future research will be to explore whether the risk factors identified in this study are connected to a possible reservoir of SDSD in sheep flocks. Investigations of bacterial sources on the animals and in the environment to enhance our knowledge of disease dynamics and bacterial transmission could pave the way for effective strategies for treatment, control and prevention of infectious arthritis in lambs.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13028-020-00561-z.

Additional file 1. Questionnaire, translated version.

Acknowledgements
The farmers who tested and responded to the questionnaire are gratefully acknowledged for their contributions. Petter Hopp, Synnøve Vatn, Thea Blystad Klem and Tormod Mørk are acknowledged for their inputs to the questionnaire.

Prior publication
The data have not been published previously.

Authors' contributions
MS performed data analyses, data interpretation and had the main responsibility for writing the manuscript. CW contributed to data analyses, data interpretation and writing the manuscript. TT contributed to planning the study, developing and publishing the questionnaire, data interpretation and writing of the manuscript. VT contributed to planning the study, developing and publishing the questionnaire, data interpretation and writing of the manuscript. CP contributed to planning the study, developing the questionnaire and writing the manuscript. AHK contributed to planning the study, developing the questionnaire and writing the manuscript. HJJ was responsible for planning the study, developing and publishing the questionnaire, data interpretation and writing the manuscript. All authors read and approved the final manuscript.

Funding
This research was conducted as a part of the project "Increasing sustainability of Norwegian food production by tackling streptococcal infections in modern livestock systems" (Project Numbers 280364 and 288917), supported by the Norwegian Research Council, the Norwegian Meat and Poultry Research Centre and TINE SA.

Availability of data and materials
The dataset is available from the corresponding author on reasonable request.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
[1] Norwegian Veterinary Institute, Ullevålsveien 68, 0454 Oslo, Norway. [2] TINE SA, Postboks 7, 0901 Oslo, Norway. [3] Norwegian Meat and Poultry Research

Centre, Lørenveien 38, 0513 Oslo, Norway. [4] Norwegian University of Life Sciences, Faculty of Veterinary Medicine, Institute for Production Animal Clinical Science, Svebastadveien 112, 4325 Sandnes, Norway.

Received: 3 July 2020 Accepted: 10 November 2020
Published online: 23 November 2020

References

1. Tømmerberg V, Tollersrud T, Salzano S, Krogh AK, Hansen B, Mikkelsen L. Outbreak of infectious arthritis in lambs. Praksisnytt. 2017;2:19–25 (**(in Norwegian)**).
2. Animalia. The Norwegian livestock industry's joint action plan on antimicrobial resistance. 2017. https://www.animalia.no/contentassets/0febbd19eb4b47338d704b18529ad253/eng_husdyrnaringas-hplan-amr-endelig-enkeltsider_220617.pdf. Accessed 20 Apr 2020.
3. Angus K. Arthritis in lambs and sheep. Practice. 1991;13(5):204–7.
4. Thompson KG. Bones and joints. In: Maxie MG, editor. Jubb, Kennedy and Palmer's pathology of domestic animals, vol. 1. 5th ed. St. Louis: Elsevier; 2016. p. 1–184.
5. Ersdal C, Jørgensen HJ, Lie KI. Acute and chronic *Erysipelothrix rhusiopathiae* infection in lambs. Vet Pathol. 2015;52:635–43.
6. Watkins GH, Sharp M. Bacteria isolated from arthritic and omphalatic lesions in lambs in England and Wales. Vet J. 1998;156:235–8.
7. Watt B, Refshauge G. An outbreak of *Streptococcus dysgalactiae* arthritis in neonatal lambs. In: Proceedings of the 3rd AVA/NZVA Pan Pacific veterinary conference. Brisbane. 2010.
8. Ridler A, Hickson R, Griffiths K, Pettigrew E, Kenyon P. Effects of *Streptococcus dysgalactiae* polyarthritis on lamb growth and mortality and risk factors for disease. Small Rumin Res. 2019;177:25–8.
9. Lacasta D, Ferrer LM, Ramos JJ, Loste A, Bueso JP. Digestive pathway of infection in *Streptococcus dysgalactiae* polyarthritis in lambs. Small Rumin Res. 2008;78:202–5.
10. Watkins GH. Arthritis. In: Aitken ID, editor. Diseases of sheep. 4th ed. Oxford: Blackwell Science; 2007. p. 288–97.
11. Blakemore F. Joint-ill (polyarthritis) of lambs in East Anglia. Vet Rec. 1939;51:1207–20.
12. Veterinary Laboratories Agency. Veterinary Laboratories Agency's surveillance report. Vet Rec. 2008;158:639–42.
13. Veterinary Laboratories Agency. Veterinary Laboratories Agency's surveillance report. Vet Rec. 2011;168:401–3.
14. Veterinary Laboratories Agency. Veterinary Laboratories Agency's surveillance report. Vet Rec. 2007;160:783–6.
15. Rutherford S-J, Jeckel S, Ridler A. Characteristics of sheep flocks affected by *Streptococcus dysgalactiae* arthritis. Vet Rec. 2015;176:435.
16. Statistics Norway. Statistics for agriculture, forestry, hunting and fishing. https://www.ssb.no/en/jord-skog-jakt-og-fiskeri/statistikker/jordhus. Accessed 31 Mar 2020.
17. Norwegian sheep recording system. Production results in Norwegian sheep herds reporting to the Norwegian sheep recording system, yearly report 2018. https://www.animalia.no/globalassets/sauekontrollen---dokumenter/arsmelding-sau-2018.pdf. Accessed 22 Apr 2020.
18. Animalia. Report on Norwegian meat and egg production, 2019. https://www.animalia.no/contentassets/3dce35cde68a47b091097fa8c6ec2dd5/kjottets-tilstand-2019.pdf. Accessed 20 Apr 2020 **(In Norwegian)**.
19. Regulation on small ruminant welfare. FOR-2005-02-18–160. https://lovdata.no/dokument/SF/forskrift/2005-02-18-160. **(in Norwegian)**.
20. European Parliament and Council of European Union (2016) Regulation (EU) 2016/679. https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32016R0679&from=EN. Accessed 01 Oct 2020.
21. Dohoo I, Martin W, Stryhn H. Veterinary epidemiologic research. 2nd ed. Charlottetown: VER Inc; 2014.
22. Edwards D, Johnston A. Welfare implications of sheep ear tags. Vet Rec. 1999;144:603–6.

Publisher's Note

Supplementary material Paper I

Additional file 1. Questionnaire, translated version, available at

https://doi.org/10.1186/s13028-020-00561-z

II

Veterinary Microbiology 262 (2021) 109221

Contents lists available at ScienceDirect

Veterinary Microbiology

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

Molecular detection and genotype characterization of *Streptococcus dysgalactiae* from sheep flocks with outbreaks of infectious arthritis

Marit Smistad [a,b,*], Tore Skeidsvoll Tollersrud [c], Lars Austbø [a], Davide Porcellato [d], Cecilia Wolff [a], Basma Asal [a], Clare J. Phythian [e], Oddvar Oppegaard [f], Hannah Joan Jørgensen [a,**]

[a] Norwegian Veterinary Institute, P.O. Box 64, 1431, Ås, Norway
[b] TINE SA, P.O. Box 7, 0901, Oslo, Norway
[c] Norwegian Meat and Poultry Research Centre, Lørenveien 38, 0513, Oslo, Norway
[d] Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, P.O. Box 5003, N-1432, Ås, Norway
[e] Faculty of Veterinary Medicine, Institute for Production Animal Clinical Science, Norwegian University of Life Sciences, Svebastadveien 112, 4325, Sandnes, Norway
[f] Department of Medicine, Haukeland University Hospital, Norway

ARTICLE INFO

Keywords:
Bacterial reservoir
Joint ill
MLST
Ovine
Whole genome sequencing

ABSTRACT

Outbreaks of infectious arthritis in young lambs are a growing concern for the Norwegian sheep industry. In other countries, *Streptococcus dysgalactiae* subspecies *dysgalactiae* (SDSD) is a frequent cause of such outbreaks. The objectives of this study were to investigate the causes of outbreaks of infections arthritis in Norwegian sheep flocks, and describe the sources, colonization patterns and genetic diversity of SDSD in affected and healthy sheep flocks. Almost 2000 samples from joints, animal body sites and the indoor environment were analysed by qPCR and culturing for SDSD, which was detected in 27 of 30 flocks. The proportion of positive samples was greater in outbreak flocks compared to healthy flocks. Altogether, SDSD was detected in 48 % of the samples from lambs, 27 % of the samples from ewes and 48 % of environmental samples. A relatively high proportion (67 %) of ear tag wounds were SDSD positive. These wounds may provide a port of entry for SDSD.

Whole genome sequencing revealed a clonal distribution of SDSD-isolates, and identified four different multi locus sequence types (STs), among which two STs, ST454 and ST531, dominated. These STs were found in geographically distant flocks. ST454 was almost exclusively found in outbreak flocks.

The current study points to skin, wounds and mucous membranes of animals as the main reservoir of SDSD in sheep flocks. However, a significantly higher proportion of SDSD-positive environmental samples in outbreak flocks compared to healthy flocks suggests that also indirect transmission may play a role.

1. Introduction

Streptococcus dysgalactiae is currently divided into the subspecies *equisimilis* (SDSE) and *dysgalactiae* (SDSD). SDSE causes infections in humans and animals, while SDSD is almost exclusively isolated from animals (Vieira et al., 1998), and is particularly associated with outbreaks of infectious arthritis in young lambs (Ridler et al., 2019; Rutherford et al., 2015; Watt and Refshauge, 2010) and mastitis in dairy cows (Unnerstad et al., 2009; Vakkamäki et al., 2017).

In Norway, outbreaks of infectious arthritis (joint ill) in sheep flocks have been a growing concern in the last decade, and pilot investigations in 2018 suggested that SDSD is frequently associated with these outbreaks (results not published). Affected lambs are usually under four weeks of age and exhibit polyarthritis coupled with systemic clinical signs (Angus, 1991; Watkins, 2007).

The pathogenesis, transmission and reservoirs of SDSD relevant to outbreaks of infectious arthritis in sheep flocks are largely undiscovered. One question is whether the lambs acquire the infection from colonized ewes or from a contaminated environment. In the UK, SDSD was detected by culturing in less than 4% of vaginal swabs from ewes, and navels and oral cavity of lambs during outbreaks of infectious arthritis (Rutherford et al., 2014) indicating a small, but most likely relevant

* Corresponding author at: Norwegian Veterinary Institute, P.O. Box 64, 1431, Ås, Norway.
** Corresponding author.
E-mail addresses: Marit.smistad@tine.no (M. Smistad), Hannah.jorgensen@vetinst.no (H.J. Jørgensen).

https://doi.org/10.1016/j.vetmic.2021.109221
Received 12 April 2021; Accepted 29 August 2021
Available online 30 August 2021

animal reservoir. Few studies have investigated SDSD in the animal environment.

Under Norwegian management conditions, outbreaks of arthritis in lambs occur during indoor housing. Identified risk factors include large flock size (> 200 ewes) and plastic mesh flooring in the sheep shed (Smistad et al., 2020). The latter suggests that the floor somehow affects transmission or that it can be a reservoir of bacteria. However, in 2017 and 2018, attempts to culture SDSD from 76 environmental samples collected in 15 sheep flocks experiencing outbreaks, were unsuccessful (not published).

The navel has been suggested as a port of entry for streptococci, with unhygienic conditions in the lambing pens as a possible predisposing factor (Angus, 1991; Blakemore, 1939; Cornell and Glover, 1925). Others have suggested that the infection may be acquired *per os* with milk from ewes with mastitis (Lacasta et al., 2008), and from wounds associated with tail docking-, or castration (Lloyd et al., 2016). Lambs are not tail docked or neutered in Norway, but infected ear tag wounds were recently identified as a risk factor for outbreaks of infectious arthritis in Norwegian lambs (Smistad et al., 2020).

By culture, SDSD colonies are inconspicuous, and despite attempts to culture on a selective medium for streptococci, Rutherford et al. (2014) isolated SDSD in low frequencies. The low sensitivity of detection, particularly in samples with a rich bacterial load, might be overcome by qPCR. To our knowledge, no studies have investigated sources of SDSD in sheep flocks using qPCR-technology. Little is also known about the genetic diversity of SDSD from sheep, and whether particular genetic bacterial traits are associated with outbreaks of infectious arthritis.

A better understanding of the reservoirs of SDSD in sheep flocks and the potential mechanisms of transmission is a prerequisite for informing targeted prevention of outbreaks of infectious arthritis in sheep flocks. This study aimed to investigate bacterial causes of outbreaks of infectious arthritis in young lambs and to describe the sources, colonization patterns, genetic traits and diversity of SDSD in affected sheep flocks.

2. Methods and techniques

2.1. Source population, selection and recruitment of flocks

In 2019, Norway had approximately 14,000 sheep farms with an average flock size of 64 breeding ewes (Statistics Norway, 2019). Typically, Norwegian sheep flocks are housed during the winter and during lambing. Sheep are kept for meat and wool, and the composite crossbreed Norwegian White Sheep accounts for about 70 % of the national population. Movement of ewes between flocks is prohibited, and breeding rams can only be exchanged between flocks within the same county.

Prior to the lambing season of 2019, to recruit sheep flocks for the study, information about the project was e-mailed to 52 veterinary practices all over the country known to work with sheep. They were encouraged to contact the project group if they came across sheep flocks with an outbreak of arthritis during the lambing season. Information was also posted on a Facebook group for Norwegian production animal veterinarians with 2000 members and on the web site of the Norwegian Meat and Poultry Research Centre.

2.2. Flock visits

Sheep flocks were included if they i) had an outbreak of infectious arthritis in lambs (≥5 % of lambs affected) in 2019 or 2020, or ii) had a history of an outbreak in previous lambing seasons. In addition, a convenience sample of sheep flocks with no previous history of an outbreak was included for comparison. Farmers of 30 sheep flocks consented to participate, of which 11 had an outbreak of infectious arthritis in their flock the season of sampling.

The flocks were visited during three periods: 1) the lambing season of 2019 (April-May) (24 flocks), 2) during the indoor housing in November 2019 (10 flocks), and 3) the lambing season of 2020 (April-May) (10 flocks). Ten of the 30 flocks, were visited in more than one of the three periods.

Outbreak flocks were visited at the earliest opportunity after the outbreak was reported. Twenty-one flocks were sampled by local veterinarians following written and oral instructions, and nine flocks were visited by veterinarians in the project group. Veterinarians that visited an outbreak flock were asked to also visit at least one flock without a joint ill outbreak the current season, hereafter referred to as "non-outbreak flocks". During visits the following information was collected: flock size, previous history of outbreaks, housing type, routines for ear tagging and navel treatment. The number of lambs affected by arthritis was recorded by the farmer and reported when the lambing season had ended.

2.3. Sampling regime

The sampling regime differed slightly between the three periods. The aim of sampling during the periods 1 and 3 (lambing seasons of 2019 and 2020) was to identify bacteriological causes of arthritis and sources of SDSD during lambing. Samples were collected from body sites of ewes and lambs, and from the environment.

In outbreak flocks, affected lambs and their mothers were selected for sampling. In non-outbreak flocks, four arbitrarily selected pairs of ewes and their lambs, preferably from different pens, were sampled. Compared to 2019, sampling in 2020 included more environmental samples and joint aspirates.

Joint aspirates were only collected from one swollen joint of lambs with suspected infectious arthritis. This sampling was done as part of the diagnostic evaluation performed by visiting veterinarians and no joint aspirates were collected from clinically unaffected lambs.

The aim of sampling during period 2 (autumn) was to identify reservoirs of SDSD on breeding sheep outside the lambing season. Swabs were collected from body sites of adult ewes and six-month-old ewe lambs. The samples from all three periods are described in Tables 3, S2 and S3.

2.4. Sampling for bacteriological analyses

Swabs with 1 mL liquid Amies (E-swabs or M 40 Transystem, Copan Diagnostics inc) were used for the majority of samples, but three outbreak flocks were sampled using swabs with Amies and charcoal as transport medium.

For dry sites such as skin and dry wounds, the swab was first dipped in the transport medium before it was rolled over the surface. Vaginal and rectal swabs were collected by inserting the swab 3−5 cm into the vagina or rectum and gently rolling the swab over the rectal or vaginal wall. Joint aspirates were collected using sterile needle and syringe from warm, swollen joints following disinfection of the skin. The aspirate was transferred to a swab and any remaining material was transferred to a sterile tube.

All samples were chilled immediately and sent chilled in polystyrene boxes express overnight to the laboratory.

2.5. Bacteriological culturing

With the exception of joint aspirates, all swabs were plated on blood agar (BA) plates (Oxoid, Basingstoke, UK) with bovine blood supplemented with 0.5 g/l aesculin and Streptococcus supplement SR126 (Oxoid) with a final concentration of 2.5 mg/l oxolinic acid and 5 mg/l colistin sulfate. Suspected SDSD colonies were subcultured for purity and identified with a matrix assisted laser desorption/ionization time of flight apparatus (MALDI-ToF MS Microflex LT system, Bruker Daltonik GmbH, Bremen, Germany).

Joint aspirates were plated on BA without supplements. Plates were incubated anaerobically and aerobically (5 % C02) for 24 h. Culture negative joint aspirates were enriched in heart infusion broth (BD Bacto)

with 5% horse serum for 24 h before 10 µL was plated on BA and cultured as described above. Bacterial colonies were identified using MALDI-ToF.

Laboratory procedures differed slightly between 2019 and 2020: all samples collected in 2019 were cultured immediately upon arrival at the laboratory. In period 3 (2020), only joint aspirates were plated upon arrival. Remaining samples were analysed first by qPCR, and only the qPCR-positive samples were thawed and cultured. Between qPCR and culturing the samples were stored at −20 °C for a maximum of two weeks.

2.6. Sample preparation and extraction of DNA

DNA from all samples was extracted with a MagNA Pure 96 instrument (Roche). For samples collected on swabs and assigned for qPCR, 250 µL transport medium was mixed with Bacterial Lysis Buffer 1:1. DNA was extracted with MagNA Pure DNA and NA LV Kit (Roche) using the Pathogen Universal protocol with a 50 µL elution volume. For DNA extraction from bacterial isolates selected for whole genome sequencing, fresh bacterial colonies on BA were dissolved in 1 mL phosphate buffered saline, and 250 µl were mixed with Bacterial Lysis Buffer 1:1 and mechanically disrupted, four times for one minute, using FastPrep-24 and 2 mL Lysing Matrix B (MP biomedicals). DNA was extracted using MagNA Pure DNA and NA SV Kit (Roche). With an input of 200 µL, genomic DNA was extracted using the DNA Blood ds SV protocol optimized for double-stranded DNA and whole genome sequencing and eluted in 50 µL.

2.7. Design of two qPCR-assays for detection of SDSD

Based on sequence data from SDSD strain ATCC 27,957 and other publicly available sequence data, two independent qPCR-assays were developed to specifically detect SDSD by targeting the 16S, and the non-specific ribonucleoside hydrolase gene (*rihC*), respectively (Table 1). Jordal et al. (2015) found *rihC* to be consistently present in SDSD but absent or largely divergent in related *Streptococcus* species. The two qPCR-assays were designed to be run in a multiplex qPCR-reaction with FAM and HEX labelling of the probes.

2.8. Multiplex qPCR-analyses of samples

Real time PCR was performed using the Brilliant Multiplex qPCR Master Mix (Agilent) and an AriaMx instrument using a two-step PCR cycling protocol initiated by 10 min at 95 °C followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. In accordance with the manufacturer's protocol, PCR was prepared in 20 µL reactions including 2 µL of template and modified with and a final concentration of 0.005 % bovine serum albumin. Samples were considered qPCR-positive if the quantitation cycle (Cq) was ≤40 in either assay. Agreement between the two qPCR-assays was calculated with Cohen's kappa coefficient after dichotomizing results as positive or negative.

2.9. Genome sequencing and bioinformatics

At least one isolate from each culture-positive flock was selected for whole genome sequencing. From flocks with less than four isolates, all were sequenced. For flocks with four or more isolates, isolates from as many different sampling sites as possible were sequenced.

In total 77 bacterial genomes were sequenced, 17 of which are also described in a study comparing *Streptococcus dysgalactiae* from different species (Porcellato et al., 2021, manuscript under review). All genomes are available at DDBJ/ENA/GenBank under the BioProject PRJEB42928.

Sequencing was performed using the Illumina MiSeq platform (Illumina, San Diego, California, USA) and V3 chemistry. Genome assembly was performed for each isolate using Shovill program (https://github.com/tseemann/shovill). Contigs shorter than 1000 bp and with coverage < 3 were removed.

For MLST-assignment, the draft genome was used as input in the MLST 2.0 (cge.cbs.dtu.dk/services/MLST) using the MLST-scheme of *Streptococcus dysgalactiae* subspecies *equisimilis*.

Core genome single-nucleotide polymorphisms (SNP) were used to study phylogenetic relationships between the isolate genomes using parSNP (Treangen et al., 2014). The genome of the SDSD type strain NCTC13759 obtained from NCBI (RefSeq accession number GCF 900459065) was used as reference. Results from parSNP were converted to a core genome alignment which was used to construct a maximum likelihood tree using the Geneious software V 10.0.7 (www.geneious.com) with Jukes–Cantor distance and supported by a bootstrapping test with 100 resamples. Phylogenetic trees were visualized using the R package ggtree (Yu, 2020).

The genomes were screened for streptococcal virulence factors using Geneious prime 2020.2.4.

2.10. Statistical analyses

Data were analysed in Stata (Release 14.2, Stata Corp LLC, USA, 2015). Flock attack rates (AR) were calculated as the percentage of lambs affected by infectious arthritis per season. An AR of 5 % or more was used as cut-off for defining an outbreak flock.

Culture- and qPCR results were summarized per flock and sampling site. To test if flocks with more positive samples were more likely to be outbreak flocks, the proportion of qPCR-positive samples per sampling site was calculated for all flocks that had three or more samples per sampling site. The median proportion of positive samples was used to assess the association between "high" (above-median) or "low" (below-median) proportion of positive samples and type of flock (outbreak *vs* non-outbreak) using Fisher's exact test. Association between type of flock (outbreak *vs* non-outbreak) and sequence type was assessed using Fisher's exact test.

3. Results

3.1. Flock data

Altogether, thirty flocks were included in the study. Of these, 23 flocks were visited one sampling period, three flocks were visited two sampling periods, and four flocks were visited all three sampling periods.

The flocks were located in Northern Norway (n = 16), Western Norway (n = 9) and Eastern Norway (n = 5) (Fig. 1). Mean flock size was 256 ewes (range 69–500) for the outbreak flocks, and 274 ewes (range 28–752) for non-outbreaks flocks. Norwegian White Sheep (NWS) was the main breed (> 80 % of the ewes) in 22 flocks, six flocks had NWS in combination with other breeds and two flocks had sheep breeds other

Table 1
Primers and probes designed in this study for the qPCR-assays to detect *Streptococcus dysgalactiae* subspecies *dysgalactiae*-genes16S and *rihC*.

Name	Sequence	Final concentration	Product
Sdd-16S-F	AGGACTGGTGCTTGCACC	1,0 µM	147bp
Sdd-16S-R	TTGCACCTTTTAAATGAAARAC	1,0 µM	
Sdd-16S-P	HEX-TCCATTGTCATGCGGTATTAGCTAT-BHQ	0.5 µM	
Sdd-rihC-F	CATTGCCTACCTGACACAGC	1,0 µM	82bp
Sdd-rihC-R	TCCAGTAGCAAGGCCATCAG	1,0 µM	
Sdd-rihC-P	FAM-ACCATCCAAGAGAAGTATTTGCGGGT-BHQ	0.5 µM	

than NWS. All the flocks practiced indoor lambing. Ten of the visited flocks had most lambings on metal mesh flooring while nine flocks had a combination of different flooring types. Five, four and two flocks had lambing on deep litter, plastic mesh flooring, or other flooring types, respectively.

Of the visited flocks, 11 were visited during an outbreak of infectious arthritis (AR $\geq$ 5 % that season) in the 2019 (n = 9) and 2020 (n = 5) lambing seasons, and were classified as outbreak flocks. Three outbreak flocks were visited during an outbreak both in 2019 and 2020. The remaining 19 flocks had no or few cases (AR < 5 %) of infectious arthritis in lambs and were classified as "non-outbreak flocks".

The mean number of samples collected per flock at lambing was 43 (range 7–91). On average, four joint aspirates were obtained per outbreak flock (range 1–22). The low number of samples in some flocks was mainly due to a lack of swabs when veterinarians had to visit a flock urgently to treat affected lambs. According to the sampling protocol, 42 samples from each flock should be collected, in addition to samples from affected joints and infected ear tag wounds. The compliance with the written sampling protocol varied between the veterinarians.

3.2. Culturing and qPCR

3.2.1. qPCR validation and agreement between the two qPCR-assays

Both qPCR assays showed a PCR efficiency close to 100 % (16S: 97.3 % and *rihC:* 97.9 %), measured from 7 four-fold dilutions spanning Cq values from 20 to 30. The limit of detection was estimated to five SDSD genomes per reaction with a confidence of 95 %. A BLAST-search toward all available sequences (NCBI) showed full sequence agreement of both assays toward all available sequences from SDSD and no sequence similarity with other bacterial species that could result in unwanted amplification. In addition to verification by sequence BLAST, the qPCR-assays were validated on a panel of SDSD isolates (n = 29) belonging to at least 3 different MLST types and six of the most related *Streptococcus* species (n = 15) identified by MALDI-ToF. Both qPCR-assays were positive for all SDSD-isolates, and there was no cross-reaction of related streptococcal species (Table S1).

An agreement analysis was performed to compare the two qPCR assays using results from the 1988 samples analysed by qPCR. Of these, 546 samples were positive by both assays, 1234 were negative by both

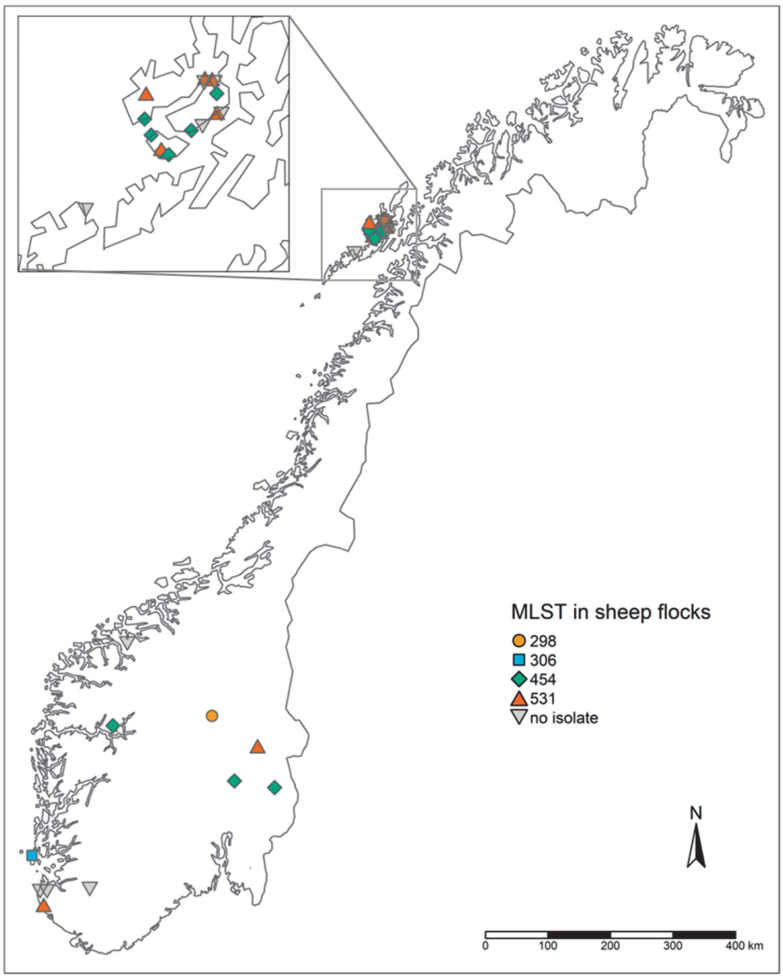


Fig. 1. Map of Norway showing the geographic location of the 30 sheep flocks with respect to the multi locus sequence types (MLST) of *Streptococcus dysgalactiae* subspecies *dysgalactiae* isolates from sheep flocks collected in 2019 and 2020.

assays, 177 were positive only by the 16S assay and 31 only by the *rihC*-assay. The Kappa agreement was 89.5 % (kappa 0.76), indicating moderate agreement. The greater sensitivity of the 16S assay, with detection of more positive samples, reflects the fact that there are five copies of the 16S-gene in the SDSD-genome. This also corresponded with a difference in the obtained Cq-values between the assays, with the 16S assay on average two cycles earlier than *rihC*.

3.2.2. Joint aspirates

Seventy-six joint aspirates from 16 flocks were cultured, whereof 30 (39 %) from 10 flocks were positive for SDSD. Other bacterial species cultured from joint aspirates were *S. aureus* (n = 5), *T. pyogenes* (n = 2) and other bacteria or contamination (n = 5). The remaining 34 (45 %) samples had no growth.

With the exception of one sample that was lost after culturing, all joint aspirates were tested by the qPCR, and 50 (66 %) were positive. SDSD was thus confirmed by culture or PCR in joint aspirates from 10 of the 11 outbreak flocks. The one flock with no SDSD-positive joint aspirate (flock O7, Table 3) had SDSD cultured from subcutaneous phlegmones of lambs affected by arthritis, and from ear tag wounds.

3.2.3. Comparison of qPCR and culture

Altogether 1901 of the totally 1988 samples were analysed by both culturing and qPCR (Table 2). The remaining 87 samples were only analysed by qPCR as they were collected in the 2020-sampling (period 3), where only qPCR-positive samples were cultured.

Of the samples analysed by both methods, 756 (40 %) were SDSD-positive by qPCR and/or culture. Of 752 qPCR-positive samples, 114 (15 %) were also culture positive, while four of 1149 qPCR-negative samples (0.3 %) were culture positive. Upon reanalyses by qPCR these four were positive (Cq-values 35.27–38.25).

Only qPCR-results were used to describe detection of SDSD in samples from sheep flocks.

3.2.4. Detection of SDSD in outbreak flocks and non-outbreak flocks

By qPCR, SDSD was detected in at least one sample from 27 of the 30 flocks. qPCR-results are shown in Tables 3 and S2, for the 2019 and 2020 sampling, respectively.

The proportion of positive samples from ear tag wounds, skin, navel, vagina and udder skin was higher in outbreak flocks compared to non-outbreak-flocks ($p < 0.05$). For samples from tonsils, nose or rectum there was no difference between outbreak flocks and non-outbreak flocks. For environmental samples, a comparison of the proportion of positive samples could only be done for the 2020-sampling, where outbreak flocks had a higher proportion of positive samples in total ($p = 0.045$). In samples collected during the autumn, results varied greatly between flocks (Table S3). There was no sampling site for which the proportion of positive samples was higher in flocks that experienced outbreaks in the lambing season of the same year, compared to non-outbreak flocks ($p > 0.05$).

3.3. Genotyping/whole genome sequencing

SDSD isolates (n = 77) from 17 sheep flocks (11 outbreak flocks and 6 non-outbreak flocks) were whole genome sequenced. Four isolates were excluded due to low quality of sequences (n = 3) or contamination (n = 1), leaving 73 genomes for analyses. The average genome size of the 73 SDSD isolates was 2.02 MB.

Four MLST-profiles were identified: STs 298, 306, 454 and 531. From flocks with more than one isolate sequenced, only one ST was identified per flock. Fifteen of the 17 flocks had isolates belonging to ST 454 (n = 8) and ST 531 (n = 7) (Tables 3 and S2). The geographical distribution of the flocks and the STs are presented in Fig. 1.

Seven of eight flocks with ST 454 were outbreak flocks (Fig. 2), while three of seven flocks with ST 531 were outbreak flocks. Fisher's exact test for the association between ST 454/531 and outbreak (yes/no) was not significant ($p = 0.1$).

Results from parSNP showed that the isolates shared ~ 75 % of the genomes with the reference strain. Of this shared genome, 19,725 bp locations were identified as SNPs. The phylogenetic tree revealed four clades that corresponded with ST (Fig. 2). A very low phylogenetic diversity was detected within isolates belonging to the same ST. Pairwise genome comparison of isolates revealed average of 57 SNPs between isolates belonging to ST 454 (median 72, range 0–215) and 56 SNPs (median 74, range 0–208) between isolates belonging to ST 531. The median number of SNPs of isolates from the same flock (always within the same ST) was between 0 and 6 for flocks with more than three isolates sequenced (n = 8, one non-outbreak flock and seven outbreak flocks). In six cases, more than one SDSD isolate from the same lamb were sequenced. For five of them the isolates differed by less than 5 SNPs. The sixth lamb had isolates differing by 30 SNPs.

The apparent association of ST 454 with outbreak flocks led us to investigate whether isolates belonging to ST 454 and ST 531 could be delineated with respect to potential virulence markers. Several notable differences were revealed, primarily linked to genes involved in bacterial adhesion: The gene encoding the adhesin DemA was present in strains of ST531, but not in ST 454. The STs also had different allerlic variants of the adhesins *fnbA* and *fnbB* (~60 % identity), as well as the *emm* gene (~70 % identity) and *srr1* (~80 % identity). Moreover, distinct genetic variants encoding immunoglobulin-binding proteins were detected, ST454 harbored MIG, whereas ST531 was associated with MAG. Two prophage-associated virulence factors, the streptodornases *sda2* and *mf4*, were present only in strains of ST531 and ST454, respectively.

Table 2
Test outcome for samples analysed for *Streptococcus dysgalactiae* subspecies *dysgalactiae* by culturing (+/-) and qPCR. By qPCR a cq≤40 was considered positive. Samples were from 30 Norwegian sheep flocks visited in 2019 and 2020.

	Sampling site	n	Test outcome qPCR/Culture				Culture positive		Culture negative	
			+/+	+/-	-/+	-/-	Median Cq	Range	Median Cq	Range
Lambs	Joint aspirates	75	29	21	1	24	24.7	15.9–36.0	28.8	21.1–39.2
	Ear tag wounds	255	51	118	0	86	21.7	13.3–32.6	32.6	18.4–38.5
	Navels	97	4	45	0	48	24.7	18.8–31.3	33.5	20.3–39.6
	Skin	87	6	44	0	37	29.0	23.6–33.7	32.7	23.0–39.9
Ewes	Vagina	211	10	40	1	160	25.7	20.2–34.4	35.4	20.7–39.8
	Nose	121	2	55	0	64	28.3	29.6–29.7	33.4	20.6–35.6
	Udder skin	199	3	72	2	122	31.5	28.7–32.6	35.5	27.7–39.0
Ewes/ lambs	Rectum	348	2	36	0	310	31.7	26.4–37.0	35.6	23.5–38.6
	Tonsils	371	2	115	0	254	28.4	20.0–36.9	35.9	28.9–39.7
Environment	Other samples	31	4	16	0	11	28.1	24.6–28.3	32.3	28.8–39.7
		106	1	76	0	29	29.9	–	31.8	25.3–38.7
	Sum	1901	114	638	4	1145	24.0	13.3–36.9	33.8	18.4–39.9

Table 3
Results from qPCR-analyses for *Streptococcus dysgalactiae* subspecies *dysgalactiae* of swabs from different sampling sites in 24 Norwegian sheep flocks visited during the lambing season of 2019. Nine flocks (O1-O9) had an attack rate (AR) ≥ 5% of lambs affected by infectious arthritis and were defined as outbreak flocks, while the remaining 15 flocks (NO1-NO15) with lower attack rates were defined as non-outbreak flocks.

				qPCR-results (n qPCR-positive/n samples)													
					Lambs						Ewes					Environ-ment	Other samples[c]
Flock	AR	Isolates (n)[b]	MLST	Total	Joint aspirate	Ear tag	Navel	Tonsil	Skin	Rectum	Tonsil	Vagina	Udder skin	Rectum	Nose		
O1	40	5	454	32/54	3/3	4/4	4/5	3/5	5/5	2/5	0/4	1/4	4/4	1/4	1/4	1/2	3/5
O2	35	7	454	39/40	1/1	6/6	3/3	3/3	3/3	0/0	3/3	3/3	3/3	2/3	3/3	6/6	3/3
O3[a]	26	9	454	28/29	5/6	11/11	1/1	0/0	2/2	0/0	0/0	2/2	2/2	0/0	0/0	5/5	0/0
O4[a]	16	5	454	46/48	1/1	12/12	11/11	2/2	2/2	1/2	3/3	3/3	3/3	3/3	3/3	2/3	0/0
O5	15	2	298	6/7	1/1	3/3	0/0	0/0	0/0	2/3	0/0	0/0	0/0	0/0	0/0	0/0	0/0
O6[a]	13	6	531	34/53	2/2	4/4	3/4	3/4	3/4	3/3	6/6	3/6	1/6	1/6	4/6	1/2	0/0
O7	12	4	531	31/50	0/6	5/5	0/0	1/1	6/6	1/1	5/5	1/5	3/4	0/5	3/5	5/6	1/1
O8	11	2	454	12/19	1/1	3/3	1/1	1/1	1/1	1/4	1/1	0/1	1/1	0/1	0/1	2/3	0/0
O9	9	8	454	9/15	3/3	3/3	2/2	0/0	0/0	1/3	0/0	0/3	0/1	0/0	0/0	0/0	0/0
NO1[a]	3	27	531	91/106	1/1	7/7	4/5	8/8	14/14	2/3	6/8	10/15	9/10	4/5	8/9	7/7	11/14
NO2	2	6	306	12/35	0/1	4/4	0/1	2/3	3/5	0/3	0/4	1/4	2/4	0/5	0/0	0/1	0/0
NO3	1	1	454	48/61	1/1	10/10	10/10	3/4	4/4	1/4	2/5	2/5	5/5	2/5	5/5	3/3	0/0
NO4	<1	0	–	1/53	0/0	0/4	0/4	1/4	0/4	0/4	0/6	0/0	0/6	0/6	0/6	0/3	0/0
NO5	<1	0	–	0/30	0/3	0/3	0/2	0/2	0/3	0/0	0/3	0/3	0/3	0/3	0/3	0/2	0/0
NO6	<1	0	–	2/47	0/0	1/3	0/4	0/4	0/4	1/4	0/5	0/5	0/5	0/4	0/5	0/3	0/1
NO7	<1	0	–	0/18	0/2	0/4	0/1	0/2	0/1	0/3	0/1	0/1	0/1	0/1	0/0	0/0	0/1
NO8	<1	0	–	9/51	0/3	1/12	3/12	0/2	0/2	1/2	2/3	0/3	0/3	0/3	1/3	1/3	0/0
NO9	<1	0	–	2/80	0/0	1/7	0/7	0/7	1/6	0/0	0/10	0/10	0/10	0/10	0/10	0/2	0/1
NO10 [a]	<1	0	–	14/18	0/0	5/6	1/1	1/1	1/1	0/0	1/1	2/4	1/1	1/1	1/1	0/0	0/1
NO11	<1	1	531	25/50	0/0	7/8	6/8	2/3	2/3	1/3	1/5	1/5	4/5	0/5	1/5	0/0	0/0
NO12	<1	0	–	1/37	0/0	0/2	0/3	0/3	0/3	0/3	0/4	0/4	0/4	0/4	0/4	0/2	1/1
NO13	<1	0	–	1/50	0/0	0/4	0/4	0/4	0/4	0/4	0/6	0/6	1/6	0/6	0/6	0/0	0/0
NO14	<1	0	–	1/42	0/0	0/3	0/3	1/3	0/3	0/3	0/5	0/5	0/5	0/5	0/5	0/2	0/0
NO15	<1	1	531	5/62	0/0	1/4	0/5	0/6	3/6	0/0	0/8	0/8	1/8	0/8	0/8	0/0	0/1
Total				449/1055	19/35	88/132	49/97	31/72	50/86	17/57	30/96	29/111	40/100	14/93	30/92	33/55	19/29
%				43	54	67	51	43	58	30	31	26	40	15	32	60	66

[a] The flock was also visited during the lambing season in 2020 (Table S2).
[b] Total number of isolates collected from the flock.
[c] Samples not requested in the sampling scheme, *e.g.* nose of lambs, wounds, abscesses.

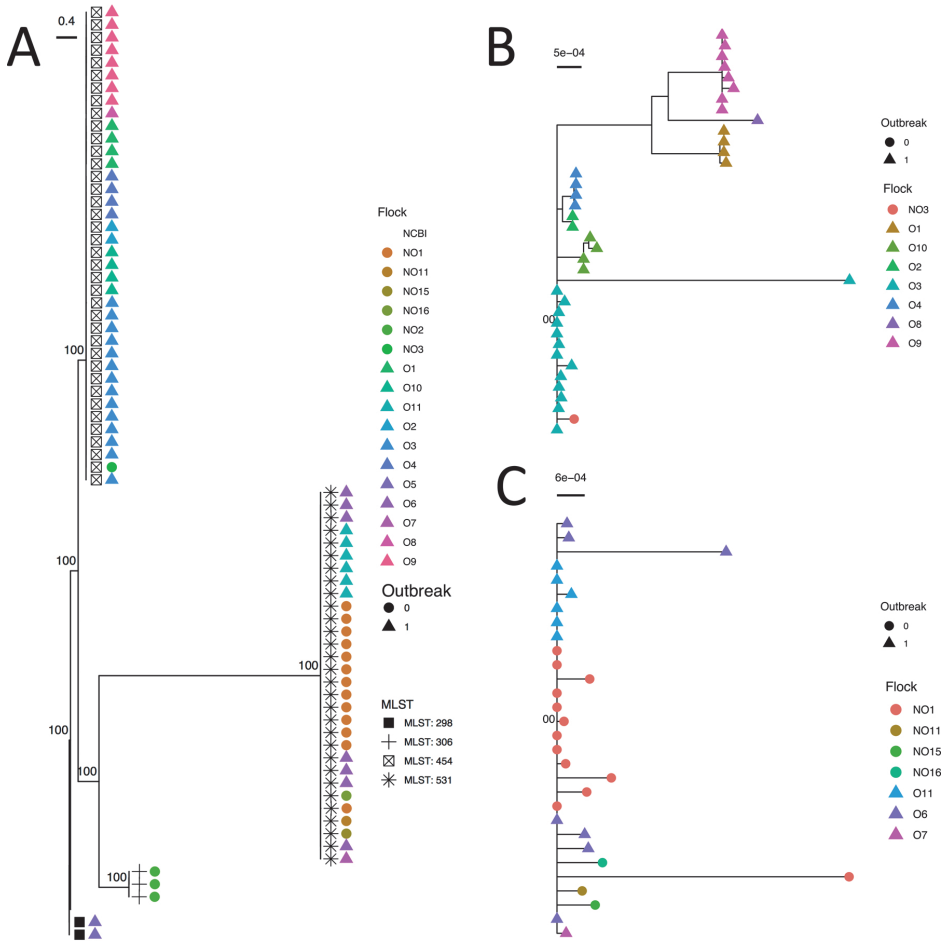


Fig. 2. Phylogenetic trees for A) all the *Streptococcus dysgalactiae* subspecies *dysgalactiae* (SDSD) isolates from Norwegian sheep flocks sequenced in this study, B) SDSD isolates belonging to MLST 454, and c) SDSD isolates belonging to MLST 531.

4. Discussion

Streptococcus dysgalactiae subspecies *dysgalactiae* was the main pathogen involved in outbreaks of infectious arthritis in young lambs in Norway.

With 90 % of the sampled flocks positive, including healthy flocks, SDSD seems to be ubiquitously present in Norwegian sheep farms. Out of 1988 samples from 30 sheep flocks, SDSD was detected in 38 % of the samples from body sites of lambs (48 %) and ewes (28 %) and from the environment (48 %). Skin, wounds and mucous membranes of animals were the major SDSD-positive body sites. The highest proportion of positive samples was found in ear tag wounds (67 %). These wounds may provide a port of entry for SDSD. Infected ear tag wounds of lambs were recently identified as a risk factor for outbreaks of infectious arthritis in Norwegian sheep flocks (Smistad et al., 2020).

Flocks suffering an outbreak of infectious arthritis in lambs had significantly higher proportions of SDSD-positive samples compared to non-outbreak flocks. The bacterial reservoir is likely to build up during an outbreak, but it could alternatively be a risk factor predisposing a flock to an outbreak. At the autumn sampling, no difference was detected between outbreak flocks and non-outbreak flocks with respect to the proportion of positive samples. This suggests that infection pressure builds up closer to or during the intensive lambing period when animal stocking density and the number of susceptible animals increases, and may also relate to management factors.

Three non-outbreak flocks had a high proportion of SDSD-positive samples, resembling the situation in outbreak flocks. All three flocks had reported outbreaks in previous lambing seasons, which raises the question as to whether immunity might develop. However, from our unpublished field data in Norway there is little to indicate that outbreaks of joint ill due to SDSD induces immunity and protection from outbreaks in the following years because many flocks experience repeated annual outbreaks. From an immunological point of view it is not likely that non-invasive colonization of ewes with SDSD would stimulate circulating anti-SDSD antibodies that can pass over in colostrum and confer protection of lambs.

It is also worth noting that the classification of flocks into outbreak flocks and non-outbreak flocks was not straight forward. We chose a relatively high cut-off for the attack rate of infectious arthritis in lambs the season of sampling, and independent of the flock's history of previous

outbreaks. The number of flocks defined as outbreak flocks would be higher if we used the 2 % cut-off as was used by Rutherford et al. (2015) or included flocks with outbreaks in the previous season. Flock sizes are smaller in Norway compared to Britain, however, and using a 5 % cut-off reduced the likelihood of misclassifying small flocks with a few sporadic cases of arthritis.

The relative importance of direct and indirect transmission routes for infection of lambs with SDSD could not be assessed in this study, but the higher proportion of culture positive samples from animals (7 %) compared to the environment (<1 %) do suggest that ewe-lamb contact as well as direct skin contact between animals in a flock are likely to be more important than indirect transmission. However, a significantly higher proportion of environmental samples were SDSD-positive in outbreak flocks compared to non-outbreak flocks. A recent study showed that greater flock size was a risk factor for outbreaks of infectious arthritis in Norwegian sheep flocks, but was not able to assess stocking density which is likely to influence direct transmission (Smistad et al., 2020). The same study showed that plastic mesh flooring was a risk factor for outbreaks, indicating a role of indirect environmental transmission. Unfortunately, few samples from plastic mesh floors were received in this study and this type of floor could not be evaluated as a source of SDSD to lambs.

Whole genome sequencing revealed four phylogenetic clades of isolates, corresponding to four different STs. Two STs (ST 454 and ST 531) dominated and were found in eight and seven flocks, respectively. Isolates from the same flock always belonged to the same ST, and a low genetic diversity was detected between isolates within the same ST and particularly between isolates from the same flock (median SNP difference ranged from 0 to 6). This is not surprising given that most of them were experiencing a disease outbreak where one bacterial variant is likely to dominate.

It was interesting that ST 454 and 531 were found in geographically distant areas because in Norway movement of sheep between farms in different counties is very restricted. The presence of genetically similar SDSD in flocks in geographically distant areas, reflect that these bacterial variants have been present in Norwegian sheep flocks over time and are most likely naturally associated with sheep, possibly as commensal bacteria. Among the ST 454 isolates, a geographical subclade was identified in isolates from the north of Norway (not shown). This is likely to be associated with closer contact between flocks in one region through pasture, and circulation of breeding rams within the same county.

From the fact that seven of the eleven outbreak flocks and only one non-outbreak flock had ST 454 it could be speculated that this ST has a higher pathogenic potential in lambs compared to ST 531. The one ST 454-positive non-outbreak flock had been vaccinated with an experimental SDSD-vaccine, due to previous outbreaks. The vaccine was tested in twenty farms in Norway in 2019, 2020 and 2021, and was a whole cell vaccine based on SDSD-ST454-strains (Tollersrud et al., not published). ST 531, however, was found in outbreak flocks (n = 3) and non-outbreak flocks (n = 4). Several distinct genomic features were identified in ST 454 and 531, predominantly with respect to bacterial adhesins. Allelic differences were detected in genes mediating binding to fibronectin (*fnbA*, *fnbB)*, fibrinogen (*demA*, the *emm*-gene) and epithelial cells (*srr1*). Host cell attachment is a pivotal step in the pathogenesis of bacterial infections, and the repertoire of adhesins may modulate virulence and tissue tropism in other streptococcal species (Esberg et al., 2017). The impact of allelic variation on the binding properties of SDSD warrants further investigation. ST 454 was also equipped with the gene encoding macroglo bulin/immunoglobulin binding protein MIG, which confers resistance to phagocytosis by bovine neutrophils, possibly through binding of IgG and IgA in bovine serum (Song et al., 2001). It is possible that it plays a role in immune evasion in invasive SDSD-infections in sheep.

Although differences in virulence genes were identified between the STs, the complexity of invasive streptococcal disease can probably not be explained through the presence or absence of single genetic factors. Three of the four STs identified in this study were found in outbreak flocks, and it is likely that multiple genes, also genes common to these STs, are required from initial infection/colonization to invasive disease.

In this study, the development and application of two qPCR assays to detect SDSD improved the sensitivity of detection by 83 % compared to culturing. Two different qPCR assays were developed and both were able to accurately detect SDSD in all sample types, although the 16S qPCR assay was more sensitive (97 % *vs* 77 %). This is not surprising given the higher copy number of the target gene. In the future it may be sufficient to use this assay alone.

Because sampling in this study was largely performed during the lambing season, and recruitment of outbreak flocks was done on short notice, a total of 13 different veterinarians were used for sampling and observations. To reduce the effect of possible bias introduced by the relatively high number of veterinarians, we asked them all also to visit at least one control flock. Eight of them visited both an outbreak flock and a non-outbreak flock. The main challenge of using private veterinarians during a busy lambing season was the varying compliance with the sampling protocol. By comparing proportions of positive samples rather than flock level frequencies we aimed to reduce any bias resulting from varying number of samples per farm. Nonetheless, some flocks with many samples at some sampling sites, *e.g.* NO1 and O3 (Table 3), contributed more to the total proportion of positive samples. These proportions should therefore be interpreted with care.

5. Conclusions

SDSD is ubiquitously present in Norwegian sheep flocks and is the main pathogen involved in outbreaks of infectious arthritis in young lambs. Overall, almost half of the samples from lambs and one third of samples from ewes were SDSD-positive. A higher proportion of samples from outbreak flocks were positive compared to samples from non-outbreak flocks. Whole genome sequencing revealed two major sequence types (ST 454 and ST 531) among the isolates, and although not statistically significant, ST 454 appeared to be associated with outbreak flocks. Future studies should examine management and environmental factors that influence bacterial transmission and the build-up of a bacterial reservoir in sheep flocks. A possible differences in pathogenic potential between SDSD sequence types also remain to be elucidated, and could influence the choice of strains for vaccine development.

Funding

This research was conducted as a part of the project “Increasing sustainability of Norwegian food production by tackling streptococcal infections in modern livestock systems”. The work received financial contributions from the Norwegian Agricultural Agreement Research Fund and the Norwegian Research council (grant numbers 280364 and 288917), the Norwegian Meat and Poultry Research Centre and TINE SA. The Norwegian Meat and Poultry Research Centre was involved in study design, interpretation, writing and submission of the article for publication.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank the participating farmers and the veterinarians who contributed to sample collection. We also acknowledge Elin Johanne Trettenes and Annette Hegermann Kampen at the Norwegian Veterinary Institute for their valuable contributions.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2021.109221.

References

Angus, K., 1991. Arthritis in lambs and sheep. In Pract. 13, 204–207.
Blakemore, F., 1939. Joint-ill (Polyarthritis) of lambs in East Anglia. Vet. Rec. 51, 1207–1220.
Cornell, R., Glover, R., 1925. Joint ill in lambs. Vet. Rec. 5, 833–839.
Esberg, A., Sheng, N., Mårell, L., Claesson, R., Persson, K., Borén, T., Strömberg, N., 2017. *Streptococcus* mutans adhesin biotypes that match and predict individual caries development. EBioMedicine 24, 205–215.
Jordal, S., Glambek, M., Oppegaard, O., Kittang, B.R., 2015. New tricks from an old cow: infective endocarditis caused by *Streptococcus dysgalactiae* subsp. *Dysgalactiae*. J. Clin. Microbiol. 53, 731–734. https://doi.org/10.1128/JCM.02437-14.
Lacasta, D., Ferrer, L.M., Ramos, J.J., Loste, A., Bueso, J.P., 2008. Digestive pathway of infection in *Streptococcus dysgalactiae* polyarthritis in lambs. Small Rumin. Res. 78, 202–205.
Lloyd, J., Kessell, A., Barchia, I., Schröder, J., Rutley, D., 2016. Docked tail length is a risk factor for bacterial arthritis in lambs. Small Rumin. Res. 144, 17–22.
Porcellato, D., Smistad, M., Skeie, S.B., et al., 2021. Whole genome sequencing reveals possible host species adaptation of *Streptococcus dysgalactiae*. Sci Rep 11 (17350). https://doi.org/10.1038/s41598-021-96710-z.
Ridler, A., Hickson, R., Griffiths, K., Pettigrew, E., Kenyon, P., 2019. Effects of *Streptococcus dysgalactiae* polyarthritis on lamb growth and mortality and risk factors for disease. Small Rumin. Res. 177, 25–28.
Rutherford, S.J., Rycroft, A.N., Ridler, A.L., 2014. Sources of *Streptococcus dysgalactiae* in English and Welsh sheep flocks affected by infectious arthritis (joint ill). Vet. Rec. 174, 579.
Rutherford, S.-J., Jeckel, S., Ridler, A., 2015. Characteristics of sheep flocks affected by *Streptococcus dysgalactiae* arthritis. Vet. Rec. 176, 435-435.
Smistad, M., Wolff, C., Tollersrud, T., Tømmerberg, V., Phythian, C., Kampen, A.H., Jørgensen, H.J., 2020. Flock-level risk factors for outbreaks of infectious arthritis in lambs, Norway 2018. Acta Vet. Scand. 62, 1–11.
Song, X.-M., Perez-Casal, J., Bolton, A., Potter, A.A., 2001. Surface-expressed mig protein protects *Streptococcus dysgalactiae* against phagocytosis by bovine neutrophils. Infect. Immun. 69, 6030–6037.
Statistics Norway, 2019. Statistics for Agriculture, Forestry, Hunting and Fishing. Vol 2020https://www.ssb.no/en/jord-skog-jakt-og-fiskeri/statistikker/jordhus, accessed 31.03.2021.
Treangen, T.J., Ondov, B.D., Koren, S., Phillippy, A.M., 2014. The Harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes. Genome Biol. 15, 1–15.
Unnerstad, H.E., Lindberg, A., Waller, K.P., Ekman, T., Artursson, K., Nilsson-Öst, M., Bengtsson, B., 2009. Microbial aetiology of acute clinical mastitis and agent-specific risk factors. Vet. Microbiol. 137, 90–97.
Vakkamäki, J., Taponen, S., Heikkilä, A.-M., Pyörälä, S., 2017. Bacteriological etiology and treatment of mastitis in Finnish dairy herds. Acta Vet. Scand. 59, 1–9.
Vieira, V., Teixeira, L., Zahner, V., Momen, H., Facklam, R., Steigerwalt, A., Brenner, D., Castro, A., 1998. Genetic relationships among the different phenotypes of *Streptococcus dysgalactiae* strains. Int. J. Syst. Bacteriol. 48, 1231–1243.
Watkins, G., 2007. Arthritis. Diseases of Sheep, 4th ed. Blackwell Science, Oxford, pp. 288–291.
Watt, B., Refshauge, G., 2010. An outbreak of *streptococcus dysgalactiae* arthritis in neonatal lambs. Proceedings of the 3rd AVA/NZVA Pan Pacific Veterinary Conference.
Yu, G., 2020. Using ggtree to visualize data on tree-like structures. Curr. Protoc. Bioinformatics 69, e96.

Supplementary material (Paper II)

Table S1: Isolates of Streptococcal species tested to evaluate specificity of the two qPCR-assays.

Species	Cq 16S	Cq RihC	Isolates (n)
S. agalactiae	No Cq	No Cq	3
S. canis	No Cq	No Cq	2
S. dys	14,7	16,8	29
S. dys equisimilis	No Cq	No Cq	3
S. equi equi	No Cq	No Cq	2
S. equi zooepid.	No Cq	No Cq	2
S. uberis	No Cq	No Cq	3

Table S2. Frequency of qPCR-detection of *Streptococcus dysgalactiae* subspecies *dysgalactiae* in samples collected during the lambing season of 2020 from five outbreak flocks (Attack rate (AR) >5 %) and 5 non-outbreak flocks.

				qPCR-results (n qPCR-positive/n samples)							
Flock	AR	Total	Isolates (n)[c]	MLST	Joint aspirates	Ear tag wounds	Nose	Floor	Interior fittings	Water troughs	Other env. sites
O3[ab]	30-40	46/48	12	454	12/14	7/7	13/13	8/8	2/2	2/2	2/2
O4[ab]	18	13/22	0	-	2/2		1/6	6/8	0/2	2/2	2/2
O6[ab]	9	12/17	5	531	6/11	3/3	2/3				
O10[b]	39	25/29	6	454	7/8	7/7	5/5	2/3	1/2	2/2	1/2
O11[b]	50	25/26	8	531	4/5	5/5	3/3	6/6	2/2	1/1	4/4
NO1	<1	5/13	0	-			2/4	0/4	0/1	1/2	2/2
NO10	<1	0/20	0	-		0/3	0/6	0/3	0/2	0/2	0/4
NO16	<1	0/13	0	-			0/3	0/5	0/2	0/2	0/1
NO17	<1	1/24	0	-			0/6	0/8	0/2	0/2	1/6
NO18	<1	0/19	0	-			0/6	0/8	0/2	0/2	0/1
Total		127/231			31/40	22/25	27/55	22/53	5/17	8/17	12/24

[a]outbreak in lambing season of 2019

[b]outbreak in lambing season of 2020

[c]Number of *Streptococcus dysgalactiae* subspecies *dysgalactiae* isolates collected from the farm

Table S3 Number of samples qPCR-positive for *Streptococcus dysgalactiae* subspecies *dysgalactiae* from the autumn sampling in 2019 from 10 flocks where 10 six months old lambs were sampled from tonsils, ear tag lesion and rectum, and 10 ewes were sampled from tonsils, rectum, udder skin and vagina.

Flock	Total	Number of isolates[c]	qPCR-results (n qPCR-positive/n samples)				
			Tonsil	Ear tag lesion	Rectum	Vagina	Udder skin
O1[a]	17/70	0	0/20	10/10	1/20	2/10	4/10
O3[ab]	23/70	0	10/20	10/10	0/20	1/10	2/10
O4[ab]	30/70	0	15/20	10/10	0/20	1/10	4/10
NO1	10/71	0	0/21	5/10	2/20	0/10	3/10
NO10	29/72	0	4/20	6/10	1/20	8/11	10/11
NO14	8/69	0	3/21	5/11	0/19	1/9	0/9
NO16	16/70	1	6/20	2/10	1/20	2/10	5/10
NO17	20/72	0	7/21	10/10	1/20	1/11	1/10
NO18	0/69	0	0/20	0/10	0/19	0/10	0/10
NO19	25/69	0	11/20	2/10	1/19	5/10	6/10
Total	178/702		56/203	60/101	7/197	21/101	35/100
%	25		28	59	3	21	35

[a]outbreak the same year (2019)

[b]outbreak the subsequent year (2020)

[c]Number of *Streptococcus dysgalactiae* subspecies *dysgalactiae* isolates collected from the farm

III

J. Dairy Sci. 105:3574–3587
https://doi.org/10.3168/jds.2021-21471

Streptococcus dysgalactiae ssp. *dysgalactiae* in Norwegian bovine dairy herds: Risk factors, sources, and genomic diversity

M. Smistad,[1,2*] H. Kaspersen,[1] F. V. Franklin-Alming,[1] C. Wolff,[1] L. Sølverød,[2] D. Porcellato,[3] E. Trettenes,[1] and H. J. Jørgensen[1]
[1]Norwegian Veterinary Institute, 1431 Ås, Norway
[2]TINE Mastitis Laboratory, N-6415 Molde, Norway
[3]Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, N-1432 Ås, Norway

ABSTRACT

Despite the importance of *Streptococcus dysgalactiae* ssp. *dysgalactiae* (SDSD) as an udder pathogen, the reservoir and epidemiological characteristics of this bacterium are largely unexplored. The aims of this study were to investigate risk factors for SDSD intramammary infections (SDSD-IMI) in Norwegian bovine dairy herds, identify sources of SDSD on animals and in the environment, and elucidate the genetic diversity of SDSD isolates. Data from herd recordings and a questionnaire were used to investigate herd-level risk factors for SDSD-IMI in 359 freestall dairy herds. Seven herds with a suspected high prevalence of SDSD-IMI were visited to sample extramammary sources (e.g., skin, wounds, mucous membranes, and freestall environment). Bacterial isolates were whole-genome sequenced to investigate the distribution of SDSD genotypes within herds and to assess the phylogenetic relationship between SDSD isolates from 27 herds across Norway. Risk factors for high incidence of SDSD-IMI in freestall dairy herds were related to housing, including closed flooring in alleys and rubber mats in cubicle bases. Parlor milking was also a risk factor compared with automatic milking systems. From herd visits, a considerable proportion of extramammary samples were SDSD positive, particularly from wounds and skin of the animals and the cubicle bases. Samples from mucous surfaces (nostrils, rectum, and vagina) and water troughs were least frequently positive. Eight multilocus sequence types (ST) were identified among the sequenced isolates from 27 herds, and phylogenetic analyses revealed 8 clades corresponding to ST. No significant association was identified between sampling site (milk, body sites, and environment) and ST. In 4 of 6 herds from which 5 or more isolates were available, one ST dominated and was found in milk and extramammary samples. One ST (ST453) was found in 15 of 27 herds, which implies that this is a widely distributed and possibly a bovine-adapted strain. Findings in this study suggest that SDSD is a cow-adapted opportunist with potential for contagious transmission, and that the freestall environment is likely to play a role in transmission between cows.
Key words: intramammary infection, multilocus sequence type, mastitis, whole-genome sequencing

INTRODUCTION

Streptococcus dysgalactiae ssp. *dysgalactiae* (**SDSD**) is an important pathogen of the bovine udder, with the ability to cause severe clinical mastitis, prolonged elevated SCC, and decreased milk yield (Whist et al., 2007; Ericsson Unnerstad et al., 2009; Heikkilä et al., 2018). It is among the major causes of IMI of dairy cows in Norway and several other countries (TINE, 2020; Duse et al., 2021; Kabelitz et al., 2021), and has negative impacts on animal welfare, economy, and antimicrobial use. According to TINE Mastitis Laboratory in Norway, the proportion of milk samples positive for SDSD increased from 6% to 13% between 2002 and 2020 (TINE, 2020).

Relatively few studies have investigated risk factors for infection of the bovine udder with SDSD (**SDSD-IMI**), or the transmission pathways and pathogenicity of this bacterium. A study from 1999 reported that an elevated incidence rate of clinical mastitis caused by SDSD in Dutch dairy herds was associated with nutrition and milking technique (Barkema et al., 1999). A more recent Finnish study showed that cows milked in a milking parlor had a higher risk of SDSD-IMI compared with cows milked in other systems (Taponen et al., 2017). The authors of that study hypothesized that this could be related to the age and design of freestall barns with parlor milking.

Questions remain whether SDSD is a contagious udder pathogen, whether it is more of an opportunist,

Received October 22, 2021.
Accepted January 5, 2022.
*Corresponding author: marit.smistad@tine.no

and whether strains differ in their pathogenic potential. Although SDSD is a common cause of subclinical mastitis in Norwegian dairy cows (TINE, 2020), it can also cause severe clinical mastitis and, in our experience, can also affect herds in an outbreak-like fashion, with many IMI cases in a short period.

Using various strain-typing schemes, previous studies have suggested that the strain diversity, and hence also the epidemiology of SDSD, may differ between farms. For example, several different SDSD genotypes can be found in individual dairy farms, as might be expected for opportunistic bacteria (Gillespie et al., 1998; Wang et al., 1999; Lundberg et al., 2016; Wente and Krömker, 2020). However, the same studies also showed that in some herds one strain dominated and could be found in milk from multiple cows. This suggests transmission between cows and that some strains might be more contagious than others.

Several studies have shown that new SDSD-IMI may establish during the dry period and in heifers before calving, even if they are not housed with lactating animals (Todhunter et al., 1995; Persson Waller et al., 2009). This suggests that sources other than infected udders may play a role in transmission, but few studies have systematically investigated sources of SDSD in dairy farms. *Streptococcus dysgalactiae* ssp. *dysgalactiae* has been detected in tonsils of cattle and calves (Daleel and Frost, 1967; Cruz Colque et al., 1993), and, more recently, Lundberg detected it on the skin of one cow and a wound of another cow (Lundberg, 2015). *Streptococcus dysgalactiae* ssp. *dysgalactiae* has also been found on flies caught in the farm environment (Bramley et al., 1985; Chirico et al., 1997). To our knowledge, with the exception of SDSD-positive flies, no studies have yet detected SDSD in the environment of dairy herds.

The genetic diversity of SDSD from Norwegian dairy herds is unknown, and we do not know whether certain bacterial genotypes are more often associated with mastitis than others. Previous studies investigating between-herd diversity of SDSD have used gel-based genotyping methods (Baseggio et al., 1997; Wang et al., 1999; Lundberg et al., 2014; Wente and Krömker, 2020) or biotyping (Aarestrup and Jensen, 1996). New studies using whole-genome sequencing (**WGS**), which has higher resolution, to investigate within-herd and between-herd diversity of SDSD are warranted.

Prevention of SDSD-IMI in dairy herds should include avoiding risk factors and reducing exposure of the cow and udder to the bacterium. The aims of the present study were to identify herd-level risk factors for SDSD-IMI in Norwegian bovine dairy herds and to investigate extramammary sources of SDSD and their possible role in transmission between cows.

MATERIALS AND METHODS

Norwegian Bovine Dairy Herds and Herd Recording System

In 2020, the Norwegian bovine dairy industry consisted of 7,214 herds with an average size of 29 lactating cows, and an annual milk yield of 8,204 kg (Statistics Norway, 2020). A total of 65% of Norwegian dairy cows were housed in freestalls, and 57% of the milk was produced in automatic milking systems (**AMS**). Norwegian Red was the main breed, accounting for 92% of the national herd (TINE, 2020).

The Norwegian Herd Recording System (**NHRS**) records production parameters such as milk recordings, farm characteristics, disease treatments, and bacteriological results of milk samples submitted to the accredited TINE Mastitis Laboratory. A total of 98% of Norwegian dairy farmers report to the NHRS (TINE, 2020).

Study Design, Data Collection, and Statistical Analyses

This study consisted of 2 parts. The first was a retrospective case-control study of herd-level risk factors for SDSD-IMI, based on data from NHRS and a farmer survey. The second was a cross-sectional field study with bacteriological investigations in 7 freestall dairy herds to identify sources of SDSD, which included WGS of SDSD for genomic characterization and phylogenetic investigation.

Risk Factor Study

Herd-level data were retrieved from the approximately 8,000 farms reporting to NHRS for the period of January 1, 2017, to December 30, 2018, including barn type, milking system, herd size, and results from bacteriological testing of milk samples. Herds with $\geq$20 cows and submitting milk samples from at least 5% of the cows (n = 1,997) were defined as eligible for inclusion as cases or controls, and, from these, herds were selected as follows. First, the incidence proportion of SDSD-IMI (number of cows with SDSD-IMI per annual average total number of cows) was calculated for each herd. Following preliminary investigation of data, it was decided that herds with incidence proportions above the 90th percentile and below the 10th percentile—that is, the herds with the 10% highest or 10% lowest proportions of SDSD-IMI—should be defined as case herds and control herds, respectively. The 90th percentile cut-off for "high proportion of cows with SDSD-IMI" (cases) was

7% (range 7–27%), and the 10th percentile cut-off for "low proportion of cows with SDSD-IMI" (controls) was 0%. All the farms with no detected cases of SDSD-IMI (39%) were therefore defined as controls. In total, 984 farms (215 cases and 769 controls) fulfilled the inclusion criteria, and the survey was sent to these farmers.

For the farmer survey, a questionnaire about herd management was created in Norwegian (English translation is available upon request). The questionnaire was designed in Questback (www.questback.com), included 46 questions, and took about 10 min to complete. Most questions were closed, or semi-closed, and, where relevant, space was provided for comments. Before distribution, 3 dairy farmers tested the questionnaire to ensure clarity, and some minor adjustments were made following feedback. The survey was distributed by e-mail in November 2019 to all selected farmers. One e-mail reminder was sent after 3 wk. To encourage participation, one participant was randomly selected to win a tablet (iPad 6th gen., 2018, Apple Inc.). Questionnaire data were merged with the data retrieved from NHRS for further analyses. All data were collected and stored according to the EU General Data Protection Regulation.

Data for the risk factor study were described and summarized. Because 79% of the herds with a high proportion of SDSD-IMI were freestall herds, and many of the investigated risk factors (e.g., type of flooring, introduction of heifers to the milking department) were relevant only for freestalls, the risk factor study included freestalls only.

For the risk factor analysis, unconditional associations between the dependent variable (case or control herd) and each of the potential risk factors were screened using χ^2 or Fisher's exact tests (categorical variables) or univariable logistic regression (continuous). Variables with a P-value ≤ 0.2 (Supplemental Table S1, https://doi.org/10.5281/zenodo.5959175) were evaluated in a multivariable logistic regression model. Tabulation was used to assess collinearity between the predictors. For correlated variables, the one with the suspected highest biological relevance was selected. The model was built using manual backward elimination. Variables were removed from the model based on a likelihood ratio test at each step, with $P < 0.05$ as criterion for retention. Herd size was forced into the model as a potential confounder to milking system. Biologically plausible interaction terms between the main effects were tested one at a time in the final model. The fit of the model was evaluated with the Hosmer-Lemeshow goodness of fit test (Dohoo et al., 2014). Data were analyzed in Stata (Release 14.2, Stata Corp. LLC, 2015).

Visits and Sampling in 7 Bovine Dairy Herds

Based on the results of the risk factor study, 7 freestall dairy farms located in Eastern Norway, designated A through G, were selected for a visit between January and March 2020. Two of the farms (A and G) were selected because they had SDSD as the main finding in milk samples the year before the visit (2019), as seen in NHRS data. The remaining 5 herds were selected based on increasing bulk milk SCC the month before the visit but with unknown prevalence of SDSD-IMI. The herds differed with respect to milking system (AMS or parlor), AMS type (DeLaval and Lely), and flooring in alleys (slatted or closed).

A sampling protocol was developed for farm visits. Because culturing of SDSD from contaminated samples is laborious (Smistad et al., 2021), the upper limit of samples for each visit was 90. Samples were collected from milk, animal body sites, the stall environment, and milking machine. Quarter milk samples were obtained by standard aseptic technique from the 10 cows with the highest SCC at the last milk recording before the visit.

Swabs with 1 mL of liquid Amies (E-swabs or M 40 Transystem, Copan Diagnostics Inc.) were used for sampling of body sites and environment. Swab samples from the same 10 cows, selected as previously described, were obtained from the skin between the udder and the thigh, hereafter termed "udder skin," and teat skin. Any visible hock lesions or other wounds were also sampled from the same 10 animals, and if fewer than 10 of these cows had wounds, wound swabs from other cows in the milking department were collected so that at least 10 wound samples were obtained from each herd. In addition, the vagina, nose, and rectum were sampled from the first 5 of the 10 cows. Vaginal and rectal swabs were collected by inserting the swab 3 to 5 cm into the vagina, nostril, or rectum, and gently rolling the swab over the mucosa.

Swab samples from cubicles were obtained from 8 different sites of the freestall in each farm. From the milking machine, swabs were collected from the liners, milk tubes, floor, and interior fittings in all herds, as well as the top of the laser or camera and the premilking teat cleaning system in the AMS herds. Swab samples were also collected from water troughs in the milking department and the cubicle bases in the calving pens.

For dry sites, the swab was first dipped in the transport medium before it was rolled over the sample area. The samples were chilled immediately and transported to the laboratory in polystyrene boxes with cooling. In addition, hock lesion scoring was performed for at

least 20 cows (or all cows if <20 cows in the herd), using score 0 (no lesion, or hairless patches <2 cm in diameter), 1 (hairless patches >2 cm in diameter, or scars >3 mm), or 2 (areas of skin damage >2 cm in diameter).

Sample Preparation and Analyses of Samples

All milk samples were cultured upon arrival at the laboratory according to standard procedures (Hogan et al., 1999). Thereafter, 250 μL from each quarter milk sample of each cow were mixed to a composite sample (cow sample), which was analyzed by quantitative (**q**) PCR, as described herein.

Samples from body sites and the environment were first screened by qPCR, and only qPCR-positive samples were cultured. The qPCR was performed upon arrival at the laboratory, and samples were thereafter frozen at −20°C for up to 2 mo before culturing.

The qPCR detected the SDSD-specific genes *rihC* and 16S (Smistad et al., 2021). Briefly, DNA was extracted from 250 μL of transport medium using MagNA Pure DNA and and NA LV Kit (Roche), and real-time PCR was performed using the Brilliant Multiplex qPCR Master Mix and an AriaMx instrument (both from Agilent).

Swab samples with a qPCR cycle quantity ≤40 were cultured. The swabs in transport medium were thawed and plated on blood agar plates (Oxoid) with bovine blood supplemented with 0.5 g/L esculin and *Streptococcus* supplement SR126 (Oxoid), with final concentrations of 2.5 mg/L oxolinic acid and 5 mg/L colistin sulfate. The plates were incubated anaerobically at 37°C for 24 h. Putative SDSD colonies were identified with MALDI-TOF (Microflex LT System, Bruker Daltonics).

Genome Sequencing of SDSD Isolates and Bioinformatics

DNA Extraction and Sequencing. All isolates obtained from the 7 herd visits (A–G), as well as 2 isolates collected in 2019 from milk samples from herds A and G, were sequenced. In addition, 20 isolates from quarter milk samples (from 20 different herds) were arbitrarily selected from a collection of SDSD isolates obtained during 2019 at the TINE Mastitis Laboratory. The isolates had been collected by freezing the first SDSD isolates obtained from clinical mastitis (n = 5) and subclinical mastitis (n = 5) every month between April and November 2019. Of the 90 sequenced isolates, 20 have been described previously (Porcellato et al., 2021). All genomes are available at DDBJ/ENA/GenBank under the BioProject PRJEB42928.

DNA was extracted using MagNA Pure DNA and the NA SV Kit (Roche), using an input of 200 μL, and the DNA Blood ds SV protocol optimized for double-stranded DNA and WGS. The template was eluted in 50 μL of the kit elution buffer.

Genomic DNA was quantified using a Qubit 3.0 fluorometer (Life Technologies) normalized to 0.2 ng/μL. The sequencing library was prepared using the Nextera XT DNA Sample Prep Kit (Illumina) before sequencing on the Illumina MiSeq platform (Illumina) and V3 chemistry.

Quality Control and Assembly of Sequences. Raw reads were quality controlled and assembled using the Ellipsis pipeline (Norwegian Veterinary Institute, 2021a). FastQC (Babraham Bioinformatics, 2019a) was used for read quality control, followed by trimming with Trim-Galore (Babraham Bioinformatics, 2019b). Genome assembly was performed by Unicycler (Wick et al., 2017) version 0.4.8 in normal mode. Genome assemblies were quality checked using QUAST (Gurevich et al., 2013). Assemblies were excluded if the total length was longer than 3 Mbp or if the number of contigs exceeded 500. For multilocus sequence type (**MLST**) assignment, the draft genome was used as input in the MLST 2.0 (cge.cbs.dtu.dk/services/MLST) using the MLST scheme of *Streptococcus dysgalactiae* ssp. *equisimilis* (McMillan et al., 2010; Porcellato et al., 2021). To test whether any sequence type (**ST**) was associated with specific sampling sites (milk, body sites, environment), the frequency of each ST in each site was compared against that of other ST (as one group) using Fisher's exact test. Only those ST with at least 5 isolates were assessed. For isolates from milk, the association between ST and clinical manifestation (subclinical vs. clinical mastitis) was tested using Fisher's exact test.

Genome assemblies were checked for contaminant sequences using Kraken2 (Wood et al., 2019), version 2.0.9. Contaminant genomes were excluded if (1) the size of the contigs identified as contaminants exceeded 10% of the total genome size and (2) contigs classified as SDSD covered less than 80% of the total genome size. Average coverage was calculated for each genome assembly. Reads were mapped to their respective genome using BWA (Li and Durbin, 2009) version 0.7.17, and sorted using SAMtools (Li et al., 2009) version 1.9. BEDtools (Quinlan and Hall, 2010) version 2.27.1 was used to extract the coverage information from the BAM file, and the average nucleotide coverage in each genome was calculated. Genome assemblies with less than 30× average coverage were excluded from further analysis.

Phylogenetic Analysis. All genomes that passed quality control were included in the phylogenetic analy-

sis, performed by the ALPPACA pipeline (Norwegian Veterinary Institute, 2021b). First, the genomes were annotated using Prokka (Seemann, 2014) version 1.14.5. Then, the pangenome was predicted and core gene alignment generated by Panaroo (Tonkin-Hill et al., 2020) version 1.2.2. The core gene alignment was de-duplicated with Seqkit (Shen et al., 2016) version 0.12.0 and then used to reconstruct the phylogeny with IQ-Tree (Nguyen et al., 2015) version 1.6.12, using ultrafast bootstrap (Hoang et al., 2018) and model finder plus (Kalyaanamoorthy et al., 2017). The de-duplicated alignment was also used to calculate the pairwise SNP distances with snp-dists (https://github.com/tseemann/snp-dists) version 0.6.3. The phylogenetic tree was visualized in R (R Core Team, 2021) version 4.0.5, using the ggtree package (Yu et al., 2017, 2018; Yu, 2020) version 2.4.2.

Following inspection of the phylogenetic tree, subclades of interest were subjected to a phylogenetic analysis with a higher resolution within the ALPPACA pipeline. First, the core genome was predicted using ParSNP (Treangen et al., 2014) version 1.5.3. The alignment was de-duplicated as previously described, and recombinant areas were identified with Gubbins (Croucher et al., 2015) version 2.4.1. The recombinant areas were masked from the de-duplicated core gene alignment with Maskrc-svg (https://github.com/kwongj/maskrc-svg) version 0.5. Then, IQ-Tree reconstructed the phylogeny from the masked alignment, and snp-dists calculated the pairwise SNP distances. The trees were visualized as described earlier.

The within-farm genetic diversity was assessed for farms with 5 or more isolates sequenced, and was based on the number of different ST per farm and the SNP distances between isolates from the same ST. To evaluate the genetic diversity of SDSD between the 27 farms, 1 isolate per ST per farm was selected for comparison.

RESULTS

Descriptive Analyses and Risk Factor Study

Of the 984 farms (215 cases and 769 controls) that fulfilled the inclusion criteria, 561 farmers (57%) responded to the questionnaire. Twelve responses were excluded due to errors in herd identification, which made it impossible to match with the NHRS data. Responses from 549 farms, 132 case herds and 417 control herds, were available for descriptive analysis (Supplemental Table S1, https://doi.org/10.5281/zenodo.5959175). The median herd size was 30 cows (interquartile range 24–45 cows), and the median yearly milk yield was 8,542 kg (interquartile range 7,858–9,105 kg). Norwegian Red was the main breed in 513 (94%) of the included herds. Therefore, breed was not included in the risk factor analysis. Freestall housing was associated with case herds (χ^2 $P = 0.001$), and the multivariable analyses included only the 365 freestall herds.

A total of 28 variables were screened in the univariable analyses, whereof 6 had a $P < 0.2$ and were tested in the multivariable model (Supplemental Table S1). For the multivariable analyses, 6 observations were excluded due to missing values in the variables "cubicle base," "type of flooring," or both. The final data set used in the multivariable model included 359 herds: 102 case herds and 257 control herds. The variable "system for scraping/removing manure" was excluded due to collinearity with type of flooring.

The final logistic multivariable regression model identified milking parlor, closed floor in the alleys, and rubber mats as risk factors for being a case herd (Table 1). The interaction terms milking system × flooring type and milking system × herd size and flooring × herd size were tested but found not significant ($P > 0.05$). The model showed good fit according to the Hosmer-Lemeshow goodness of fit test, with χ^2 (8 df) $= 7.18$, $P = 0.52$.

Sources and Genotypes of SDSD

Herd characteristics of the 7 visited farms, the number of samples, and the proportions of SDSD-positive samples per farm are presented in Table 2. Farm G was organic, and the remaining 6 were conventional. Norwegian Red was the main breed on 6 of the farms. The seventh (farm A) had Norwegian Red and other breeds.

Milk samples from 74 cows were obtained (range 10–13 per farm). Of these, 13 cows had SDSD-IMI (range 0–4 per farm). Other cow-level bacterial findings were as follows: no growth (n = 19), non-*aureus* staphylococci (n = 18), *Staphylococcus aureus* (n = 9), contamination (n = 5), *Streptococcus uberis* (n = 4), *Streptococcus agalactiae* (n = 2), and other bacteria (n = 4).

Results from qPCR analyses and bacterial culture of extramammary samples are described in Table 3. The main SDSD-positive sampling sites by qPCR were wounds, teat or udder skin, and cubicle bases, whereas samples from mucous surfaces (nostrils, rectum, and vagina) and water troughs were the least frequently positive. Wound samples and samples from cubicle bases were most frequently culture positive.

Genomic Analyses

Quality Control and Contamination Screening. Quality control of altogether 90 sequenced SDSD isolates from 27 herds resulted in exclusion of 14 iso-

lates from phylogenetic analyses due to contamination (n = 10) and low coverage (n = 4). However, it was possible to determine the ST for 13 of the excluded isolates. Hence, ST was determined for 89 isolates, and 76 isolates were included in the phylogenetic analyses.

Multilocus Sequence Typing. Eight different ST were identified among the 89 isolates for which ST could be determined. Five of these were allocated to isolates from more than one farm, including isolates from farm visits and from the mastitis laboratory (Table 4). Of these, ST453 predominated, compromising 43% of the isolates, found in 15 of the 27 herds. The remaining ST were detected in 4 herds (ST 301 and 302), 3 herds (ST 306 and 531), and 1 herd (ST 454, 532, and 524). From farms A and G, sequenced isolates were collected both from the mastitis laboratory in 2019 and at farm visits in 2020. However, in both cases, the ST of the 2019 isolates were different from the 2020 isolates. Among isolates from the 7 farm visits in 2020, the numbers of ST per farm were 1 (farms A and F), 2 (farms B and D), or 3 (farm C and E). Farm G had only 1 available isolate.

Associations between sampling site (milk, body site, environment) and ST (ST 453, 302, 301, and 531 vs. all others) were tested but were found not significant (Fisher's exact $P > 0.05$).

A total of 37 isolates from milk samples from 27 herds were sequenced. Of these, 10 isolates from 10 herds were from cows with clinical mastitis. These belonged to ST 453 (n = 5), 306 (n = 2), 301 (n = 1), 531 (n = 1), and 532 (n = 1). We found no significant association between ST and clinical manifestation (clinical vs. subclinical mastitis). Both ST 302 (farms C and E) and 453 (farm D) were found in IMI from more than one cow in the same herd.

Pangenome and Phylogenetic Analyses. The pangenome analysis identified 3,017 genes in the 76 genomes. Of these, 1,505 genes were categorized as core genes and were used to reconstruct the phylogeny. In the phylogenetic tree, the median SNP distance was 10,268, with a range of 0 to 16,481. The phylogenetic tree divided into 8 clades that corresponded with ST (Figure 1). The majority of isolates within the same ST clustered closely. Exceptions were observed for one ST 453 isolate, 3 ST 301, and 1 ST 306 (Figure 1). Four of the visited farms had one dominating ST (>80% of the isolates): farms A and D (ST 453), farm B (ST 301), and farm F (ST 302).

Separate phylogenetic analyses for ST 453, 302, and 531, with the origin of the isolates (farm and type of sample) are presented in Figures 2, 3, and 4. For ST 301, one subcluster included 8 isolates from body sites (n = 7) and one from the environment of farm B. The alignment of the ST 453 core genome had an average genome coverage of 83.4%, with a median SNP distance of 81, ranging from 1 to 223 SNPs. Within ST 453, the median SNP distance between strains from different farms was 66 (range 14–168). For the ST 302 alignment, the core genome had an average coverage of 86.0%, with a median SNP distance of 91 and a range of 1 to 114 SNPs. Finally, for the ST 531 alignment, the core genome average coverage was 95.1%, with a median SNP distance of 79 and a range of 2 to 97 SNPs. Isolates from the same farm belonging to the same ST were more closely related (median core gene SNP distances between 2 and 18). There was one

Table 1. Herd variables associated with a high incidence of *Streptococcus dysgalactiae* ssp. *dysgalactiae* IMI (case herds) in a multivariable logistic regression model[1]

Variable and category	N case herds (%)	N control herds (%)	OR[2]	95% CI	*P*-value
Milking system					
AMS[3]	68 (67)	219 (85)	Referent		
Parlor	34 (33)	38 (15)	2.9	1.6–5.1	<0.001
Type of flooring[4]					
Slatted	62 (61)	204 (79)	Referent		
Closed	36 (35)	46 (18)	2.9	1.6–5.1	<0.001
Other	4 (4)	7 (3)	1.8	0.49–6.7	0.371
Cubicle bases					
Mattresses	81 (79)	171 (67)	Referent		
Rubber mats	21 (21)	86 (33)	2.5	1.4–4.4	0.003
Herd size[5]			1.0	0.99–1.02	0.616

[1]Data from the Norwegian herd recording system and a farmer survey, Norway 2017–2018. Number of herds in data = 359, all with freestall housing.

[2]Odds ratio.

[3]Automatic milking system.

[4]Floor in alleys.

[5]Annual average number of cows, included as a confounder to milking system.

Table 2. Description of 7 Norwegian freestall dairy herds visited in 2020 for sampling and results from analyses of *Streptococcus dysgalactiae* ssp. *dysgalactiae* by quantitative (q) PCR (all samples) and culturing (qPCR-positive samples)[1]

	Farm						
Herd characteristics	A	B	C	D	E	F	G
Herd size (number of cows)	50	13	34	51	46	75	41
Milking system	AMS	Parlor	AMS	AMS	AMS	AMS	AMS
Cows with hock lesions[2] (%)	55	77	85	10	65	81	60
BMSCC[3]	126,000	160,000	196,000	121,000	169,000	175,000	131,000
Milk yield[4]	9,657	6,618	8,707	8,700	8,787	8,663	8,669
Cubicle base	Mattresses	Rubber mats	Mattresses	Mattresses	Rubber mats	Rubber mats	Mattresses
Alley flooring	Slatted	Slatted	Slatted	Slatted	Slatted	Solid	Slatted
Sample type [n qPCR-positive samples/n total (n culture positive)]							
Milk[5]	1/10 (0)	3/12 (1)	3/10 (4[6])	5/10 (5)	2/10 (3[6])	3/10 (2)	1/13 (0)
Body site swab	32/60 (4)	36/48 (8)	28/52 (6)	36/53 (6)	35/51 (1)	27/48 (6)	26/58 (1)
Environmental swab	20/23 (2)	11/14 (1)	15/22 (4)	19/21 (7)	20/25 (5)	12/20 (1)	15/25 (0)

[1]With qPCR, a cycle quantity ≤40 was considered positive.
[2]Hairless patches larger than 2 cm in diameter, or lesions or skin damage >2 mm.
[3]Bulk milk SCC, 12-mo geometric mean, 2019.
[4]ECM, annual average kilograms per cow.
[5]Milk samples were analyzed by culturing at quarter level and qPCR at cow level.
[6]Two of the samples came from different quarters from the same cow.

exception to this; the 10 ST 453 isolates from farm D divided into 2 subclusters with a maximum core gene SNP distance of 152.

DISCUSSION

The current study adds some pieces to the puzzle of sources and potential transmission routes of SDSD in Norwegian bovine dairy herds. To our knowledge, it is the first study to systematically investigate risk factors for SDSD-IMI as well as bacterial sources in bovine dairy herds, and to use WGS to compare the between-herd and within-herd genomic diversity of SDSD.

Freestall housing was associated with a high incidence of SDSD-IMI in the univariable analyses. This may explain why the prevalence of SDSD-IMI has increased in Norway in the last decades (TINE, 2020), because a shift toward freestall housing has occurred following a regulatory enforced transition from tiestalls (Norwegian Ministry of Agriculture and Food, 2004). Although freestall housing systems differ, they have in common that the cows move freely and come into direct and indirect contact with each other more frequently than in tiestalls. For example, the cubicles are shared between cows, creating potential contact surfaces for transmission. Furthermore, it is more feasible to practice a strict milking order according to infection status in tiestalls than in freestalls.

In the risk factor study, which included freestalls only, several risk factors were identified for case herds, that is, herds with a higher incidence of SDSD-IMI. Farms with closed floors in alleys had an increased risk of being a case herd compared with those with slatted floors. The mechanisms of this effect are not completely clear, but it could be related to a build-up of feces and slurry between floor scrapings in these systems, or to the higher humidity in barns with closed floors, in particular if drainage is poor.

In agreement with the findings of Taponen et al. (2017), our study identified parlor milking as a risk factor for being a case herd. Taponen et al. proposed that this could be associated with barn design, which may be a plausible explanation also in Norway. Most dairy barns built after 2010 in Norway are designed with slatted floors and have AMS, whereas closed flooring and milking parlors are more common in older freestall barns or barns rebuilt from tiestall barns. Another possible explanation for the association with the milking system may be differences in the cleaning of the teat cup liners, and hence the possibility for bacterial transfer during milking. The flushing or steaming of the teat cup liners between each cow in AMS herds is rarely practiced in parlor systems in Norway and may reduce

Table 3. Frequency of detection of *Streptococcus dysgalactiae* ssp. *dysgalactiae* by quantitative (q) PCR (all samples) and culture (qPCR-positive samples) in 7 Norwegian freestall bovine dairy herds[1]

Source	N	N qPCR positive (%)	N culture positive (%)	Median cq 16S (range)
Milk	75	18 (24)	13 (17)	32.4 (24.9–38.9)
Body site	370	220 (60)	32 (9)	33 (17.1–39.6)
Wound[2]	77	71 (92)	16 (21)	31.5 (17.1–38.5)
Teat skin	70	59 (84)	9 (13)	33.9 (27.4–38.8)
Udder skin	71	59 (83)	5 (7)	34.1 (28.0–39.0)
Tonsil calf	24	12 (50)	1 (4)	32.2 (28.6–36.2)
Vagina	34	6 (18)	0 (0)	36.0 (31.8–39.6)
Rectum	58	9 (15)	0 (0)	31.8 (25.4–39.1)
Nose	36	4 (11)	1 (3)	35.7 (30.8–38.5)
Environmental source	150	112 (75)	21 (14)	33.2 (26.8–39.1)
Cubicle base	55	52 (95)	12 (22)	33.0 (26.8–39.1)
Calving pen	9	8 (89)	1 (11)	34.0 (27.7–38.8)
Milking machine[3]	60	35 (58)	6 (10)	33.1 (29.6–37.3)
Water trough	13	5 (38)	1 (8)	36.0 (30.6–37.4)
Other environmental sample[4]	13	12 (92)	1 (8)	34.6 (32.0–38.5)
Total	595	350 (59)	66 (11)	33.2 (17.1–39.6)

[1]With qPCR, a cycle quantity (cq) ≤40 was considered positive.

[2]Mainly mild hock lesions.

[3]Swabs were collected from liners, milk tubes, floor, and interior fittings of the milking system (all herds), and from the top of the laser or camera and the pre-milking teat cleaning system (automatic milking system herds).

[4]Floor, interior fittings, concentrate station.

the transmission of bacteria at milking in AMS herds (Skarbye et al., 2020).

Finally, herds with rubber mats as cubicle bases had a higher risk of being case herds compared with herds with mattresses. Rubber mats are generally more compact than mattresses and have been associated with hock lesions (Kielland et al., 2009; Ekman et al., 2018). A high proportion of wound samples (92%) were qPCR positive in this study, and more than 20% of these were culture positive, often in almost pure growth (results not shown). Rubber mats may indirectly increase the incidence of SDSD-IMI by causing hock lesions where SDSD can multiply.

To further understand the underlying mechanisms of the association between barn type and the identified risk factors for a high incidence of SDSD-IMI in herds, we visited 7 freestall herds for bacteriological sampling. The main SDSD-positive sampling sites were wounds, teat or udder skin, and cubicle bases, whereas samples from mucous surfaces (nostrils, rectum, and vagina) were the least frequently positive. The main niche of SDSD appears to be the skin of the animals (Mundt, 1982), but the high positivity rate of environmental samples suggests that animals shed bacteria to the environment and that the environment plays a role in transmission of SDSD.

The most likely sources for contamination of the environment with SDSD is milk from cows with IMI, as well as the skin and wounds of the cows. The bacterial load in milk from cows suffering SDSD-IMI can be high (Hamel et al., 2021), and milk leakage from these cows is probably an important contribution to the environmental bacterial load. Fecal shedding may be less important as a source of SDSD to the environment because a relatively low proportion of rectal samples were qPCR positive (15%), none of which were culture positive.

However, the barn environment is not necessarily a continuous reservoir of viable SDSD. In unpublished pilot studies, we were only able to retrieve SDSD by

Table 4. Distribution of *Streptococcus dysgalactiae* ssp. *dysgalactiae* multilocus sequence types in isolates from 27 Norwegian dairy herds; isolates were collected from farm visits (n = number of isolates, from 7 farms) and from routine analyses at the TINE Mastitis Laboratory, Molde, Norway (n = number of isolates, from 20 farms across Norway)

		Sequence type								
Origin of sample	Type of sample	301 (n = 11)	302 (n = 18)	306 (n = 8)	453 (n = 38)	454 (n = 2)	524 (n = 1)	531 (n = 10)	532 (n = 1)	Total (n = 89)
Farm visits	Milk	1	7	1	11	1	0	5	0	26
	Body site	7	3	3	12	0	0	1	0	26
	Environment	1	7	0	5	1	0	1	0	15
Mastitis laboratory	Milk	2	1	4	10	0	1	3	1	22

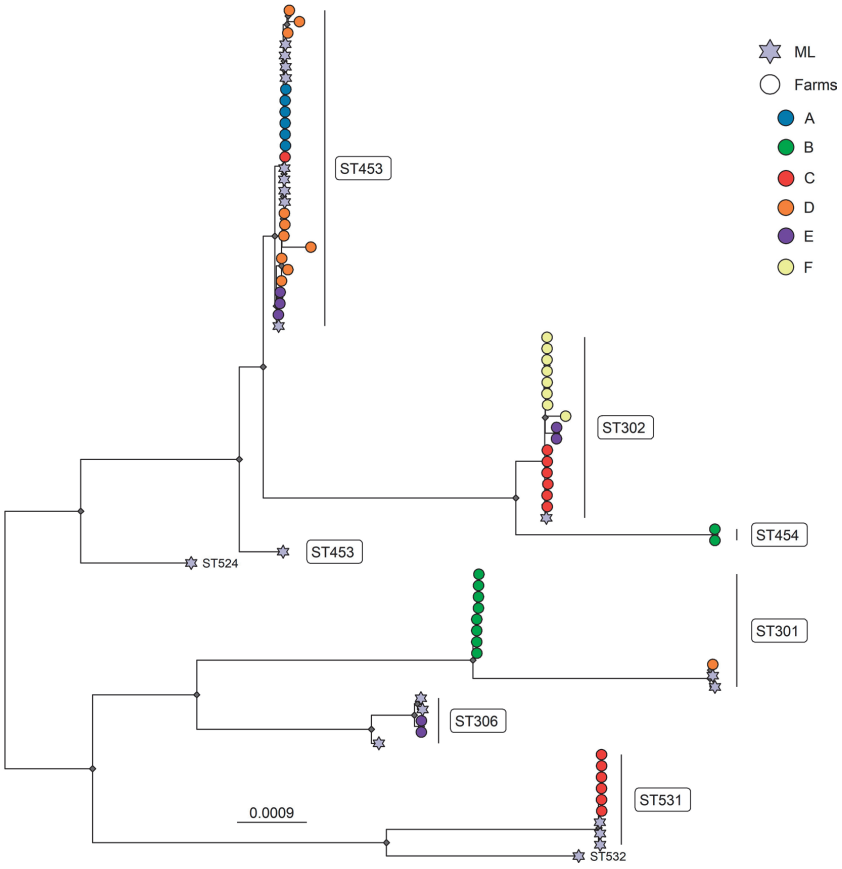


Figure 1. Maximum likelihood phylogenetic reconstruction based on an alignment of 1,505 core genes from 76 *Streptococcus dysgalactiae* ssp. *dysgalactiae* isolates. Nodes with high bootstrap support (≥95) are denoted with a diamond. Tip shapes represent sample origin: star = ML (mastitis laboratory), circle = isolates from farm visits (farms A–F). Colors represent different farms and the mastitis laboratory (lilac). ST = sequence type.

culturing for 1 d after spiking of manure taken from the floor of a bovine dairy herd and keeping it at 20°C under aerobic conditions. In contrast, we were able to culture *Strep. agalactiae* for almost 3 wk under equivalent conditions. Hence, although SDSD may be present in the environment and can transmit to cows from there, it is unlikely to multiply and survive for extended periods outside the cow. This would mean that the classical preventive measures against contagious udder pathogens, targeting infected udders, are relevant for SDSD-IMI. We propose, however, that wound prevention and increased frequency of scraping and improved hygiene in cubicle bases should be included in any program to reduce SDSD-IMI in a herd.

Whole-genome sequencing found no associations between site of isolation and ST, which indicates that bacteria can transmit from animals to the environment and vice versa. It also showed that the same SDSD strain caused IMI in multiple cows in the same herd. Similar findings have been described by others (Lundberg et al., 2016; Wente and Krömker, 2020) and can be taken as evidence of direct or indirect transmission between animals. Further studies are needed to determine whether some strains of SDSD have greater potential for contagious spread than others.

Phylogenetic analysis revealed a clonal population structure, with relatively few sequence types detected in the investigated herds and a high similarity between

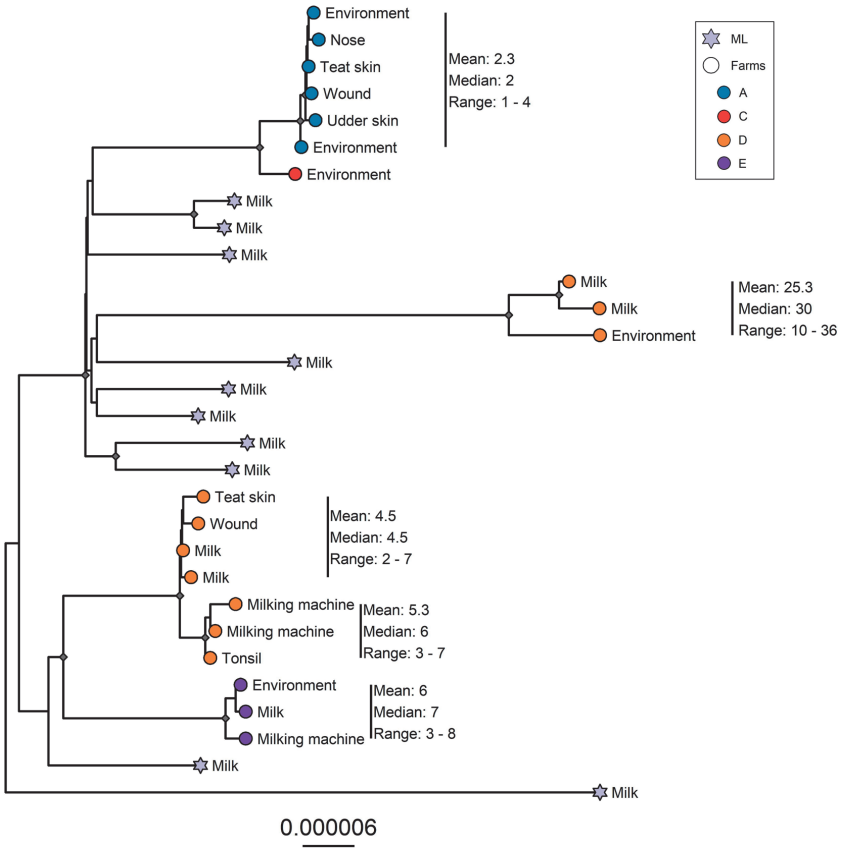


Figure 2. Maximum likelihood phylogenetic reconstruction based on the core genome alignment of the 30 *Streptococcus dysgalactiae* ssp. *dysgalactiae* sequence type 453 isolates. Nodes with high bootstrap support are denoted with a diamond. Tip shapes represent sample origin: star = ML (mastitis laboratory), circle = other (farm visits, farms A–E). Colors represent different farms and the mastitis laboratory (lilac). Tip labels represent sample type. SNP distance (number of SNPs) statistics (mean, median, range) are presented for each clade of interest.

isolates from the same ST. Furthermore, a modest number of ST were identified among the 89 sequenced SDSD isolates from 27 geographically spread farms, and one ST (ST453) was identified in 15 different herds. In a previous study, we sequenced SDSD from different species and found that SDSD delineated according to host species (Porcellato et al., 2021). By documenting that a few SDSD genotypes are more prevalent and can be found in geographically spread bovine dairy herds, our study supports the existence of bovine-adapted SDSD. Recent transmission between herds is an alternative explanation, but an epidemiological link between all these herds is unlikely. The greater SNP distance between isolates from different farms compared with isolates from the same farm also makes recent transmission between herds a less likely explanation.

An important question of this study was whether SDSD is a contagious udder pathogen or more of an opportunist. Traditionally, udder pathogens have been defined as "contagious" or "environmental," referring to the bacterial reservoirs and their principal modes of transmission (Schukken et al., 1991; Todhunter et al., 1995; Oliver et al., 2011). However, this binary classification is misleading for many important udder pathogens, including the streptococci (Klaas and Zadoks, 2018), and it may be more accurate to use the terms "environmental" or "cow-adapted" to describe the main reservoirs of the bacteria, and "opportunistic"

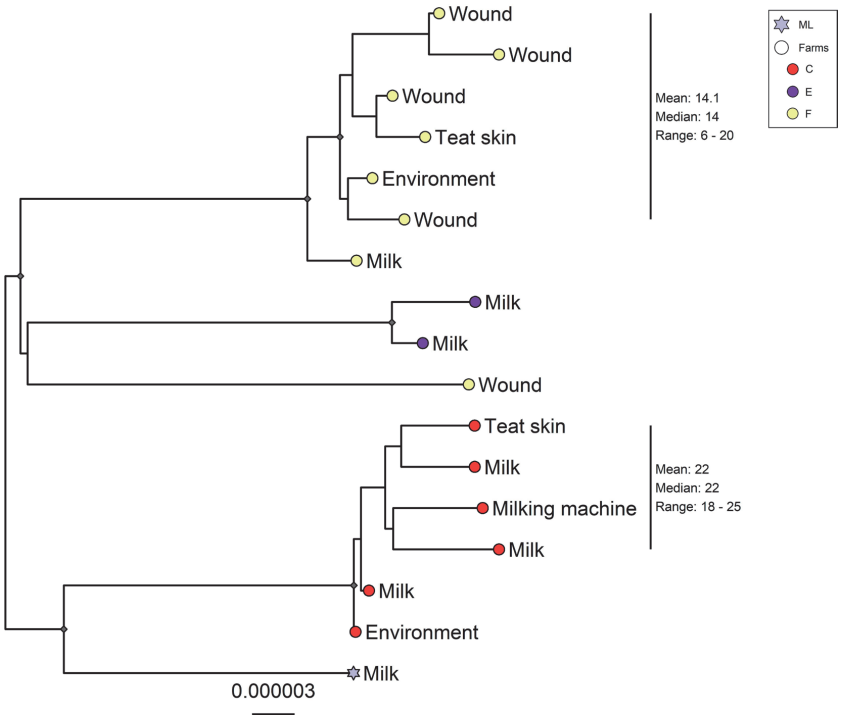


Figure 3. Maximum likelihood phylogenetic reconstruction based on the core genome alignment of the 17 *Streptococcus dysgalactiae* ssp. *dysgalactiae* sequence type 302 isolates. Nodes with high bootstrap support are denoted with a diamond. Tip shapes represent sample origin: star = ML (mastitis laboratory), circle = other (farm visits, farms C, E, and F). Colors represent different farms and the mastitis laboratory (lilac). Tip labels represent sample type. SNP distance (number of SNPs) statistics (mean, median, range) are presented for each clade of interest.

or "contagious" to describe their mode of transmission (Vanderhaeghen et al., 2015). Although our study does not fully answer these questions, it does suggest that the cow is the main niche of SDSD in dairy herds. Based on our findings, SDSD cannot unequivocally be categorized as opportunistic or contagious, but appears to be an opportunist that can also behave contagiously in certain situations

CONCLUSIONS

A high incidence of IMI caused by SDSD in Norwegian bovine dairy herds is associated with freestall housing. In addition to infected udders, sources of the bacterium include the skin and wounds of animals and the environment. This study suggests that SDSD is a cow-adapted udder pathogen with some potential for contagious spread and that the environment is one possible transmission route. The existence of SDSD on extramammary body sites is likely to contribute to the maintenance of bacterial reservoir in a herd after infected udders are treated. Therefore, wound prevention, appropriate hygiene, quality of cubicle bases, and control of IMI at dry-off are likely to be relevant measures to prevent SDSD-IMI in dairy herds.

ACKNOWLEDGMENTS

We thank the participating farmers and the team from TINE Mastitis Laboratory (Molde, Norway). Tormod Mørk (Norwegian Veterinary Institute, Ås, Norway), Anne Guro Larsgard (Geno SA, Hamar, Norway), Olav Østerås (TINE SA, Ås), and Basma Asal (Norwegian Veterinary Institute, Ås) are acknowledged for their valuable contributions. This research was conducted as a part of the project "Increasing sustainability of Norwegian food production by tackling streptococcal infections in modern livestock systems." The work re-

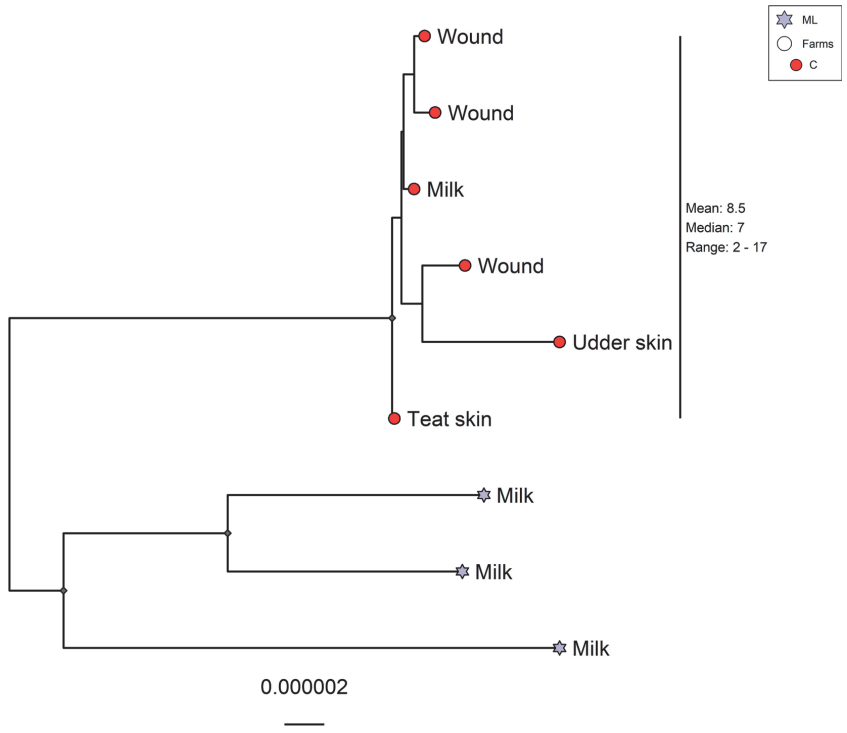


Figure 4. Maximum likelihood phylogenetic reconstruction based on the core genome alignment of the 9 *Streptococcus dysgalactiae* ssp. *dysgalactiae* sequence type 531 isolates. Nodes with high bootstrap support are denoted with a diamond. Tip shapes represent sample origin: star = ML (mastitis laboratory), circle = other (farm visits, farm C). Colors represent different farms and the mastitis laboratory (lilac). Tip labels represent sample type. SNP distance (number of SNPs) statistics (mean, median, range) are presented for each clade of interest.

ceived financial contributions from the Norwegian Agricultural Agreement Research Fund (Oslo, Norway) and the Norwegian Research council (Oslo; grant numbers 280364 and 288917), the Norwegian Meat and Poultry Research Centre (Oslo), and TINE SA (Ås). The authors have not stated any conflicts of interest.

REFERENCES

Aarestrup, F. M., and N. E. Jensen. 1996. Genotypic and phenotypic diversity of *Streptococcus dysgalactiae* strains isolated from clinical and subclinical cases of bovine mastitis. Vet. Microbiol. 53:315–323. https://doi.org/10.1016/S0378-1135(96)01221-7.

Babraham Bioinformatics. 2019a. FastQC. Accessed Jul. 1, 2021. https://www.bioinformatics.babraham.ac.uk/projects/fastqc/.

Babraham Bioinformatics. 2019b. Trim Galore. Accessed Jul. 1, 2021. https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/.

Barkema, H. W., Y. H. Schukken, T. J. G. M. Lam, M. L. Beiboer, G. Benedictus, and A. Brand. 1999. Management practices associated with the incidence rate of clinical mastitis. J. Dairy Sci. 82:1643–1654. https://doi.org/10.3168/jds.S0022-0302(99)75393-2.

Baseggio, N., P. D. Mansell, J. W. Browning, and G. F. Browning. 1997. Strain differentiation of isolates of streptococci from bovine mastitis by pulsed-field gel electrophoresis. Mol. Cell. Probes 11:349–354. https://doi.org/10.1006/mcpr.1997.0126.

Bramley, A. J., J. E. Hillerton, T. M. Higgs, and E. M. Hogben. 1985. The carriage of summer mastitis pathogens by muscid flies. Br. Vet. J. 141:618–627. https://doi.org/10.1016/0007-1935(85)90009-0.

Chirico, J., P. Jonsson, S. Kjellberg, and G. Thomas. 1997. Summer mastitis experimentally induced by *Hydrotaea irritans* exposed to bacteria. Med. Vet. Entomol. 11:187–192. https://doi.org/10.1111/j.1365-2915.1997.tb00312.x.

Croucher, N. J., A. J. Page, T. R. Connor, A. J. Delaney, J. A. Keane, S. D. Bentley, J. Parkhill, and S. R. Harris. 2015. Rapid phylogenetic analysis of large samples of recombinant bacterial whole genome sequences using Gubbins. Nucleic Acids Res. 43:e15. https://doi.org/10.1093/nar/gku1196.

Cruz Colque, J. I., L. A. Devriese, and F. Haesebrouck. 1993. Streptococci and enterococci associated with tonsils of cattle. Lett. Appl. Microbiol. 16:72–74. https://doi.org/10.1111/j.1472-765X.1993.tb00346.x.

Daleel, E. E., and A. J. Frost. 1967. Some observations on the bacterial flora of the bovine tonsil. Br. Vet. J. 123:232–236. https://doi.org/10.1016/S0007-1935(17)39953-0.

Dohoo, I., W. Martin, and H. Stryhn. 2014. Veterinary Epidemiologic Research. 2nd ed. VER Inc.

Duse, A., K. Persson-Waller, and K. Pedersen. 2021. Microbial aetiology, antibiotic susceptibility and pathogen-specific risk factors for udder pathogens from clinical mastitis in dairy cows. Animals (Basel) 11:2113. https://doi.org/10.3390/ani11072113.

Ekman, L., A.-K. Nyman, H. Landin, and K. Persson Waller. 2018. Hock lesions in dairy cows in freestall herds: A cross-sectional study of prevalence and risk factors. Acta Vet. Scand. 60:47. https://doi.org/10.1186/s13028-018-0401-9.

Ericsson Unnerstad, H., A. Lindberg, K. Persson Waller, T. Ekman, K. Artursson, M. Nilsson-Öst, and B. Bengtsson. 2009. Microbial aetiology of acute clinical mastitis and agent-specific risk factors. Vet. Microbiol. 137:90–97. https://doi.org/10.1016/j.vetmic.2008.12.005.

Gillespie, B., B. Jayarao, J. Pankey, and S. Oliver. 1998. Subtyping of Streptococcus dysgalactiae and Streptococcus uberis isolated from bovine mammary secretions by DNA fingerprinting. J. Vet. Med. B 45:585–593.

Gurevich, A., V. Saveliev, N. Vyahhi, and G. Tesler. 2013. QUAST: Quality assessment tool for genome assemblies. Bioinformatics 29:1072–1075. https://doi.org/10.1093/bioinformatics/btt086.

Hamel, J., Y. Zhang, N. Wente, and V. Krömker. 2021. Heat stress and cow factors affect bacteria shedding pattern from naturally infected mammary gland quarters in dairy cattle. J. Dairy Sci. 104:786–794. https://doi.org/10.3168/jds.2020-19091.

Heikkilä, A.-M., E. Liski, S. Pyörälä, and S. Taponen. 2018. Pathogen-specific production losses in bovine mastitis. J. Dairy Sci. 101:9493–9504. https://doi.org/10.3168/jds.2018-14824.

Hoang, D. T., O. Chernomor, A. Von Haeseler, B. Q. Minh, and L. S. Vinh. 2018. UFBoot2: Improving the ultrafast bootstrap approximation. Mol. Biol. Evol. 35:518–522. https://doi.org/10.1093/molbev/msx281.

Hogan, J., R. Gonzalez, R. Harmon, S. Nickerson, S. Oliver, J. Pankey, and K. L. Smith. 1999. Laboratory Handbook on Bovine Mastitis. National Mastitis Council.

Kabelitz, T., E. Aubry, K. van Vorst, T. Amon, and M. Fulde. 2021. The role of *Streptococcus* spp. in bovine mastitis. Microorganisms 9:1497. https://doi.org/10.3390/microorganisms9071497.

Kalyaanamoorthy, S., B. Q. Minh, T. K. F. Wong, A. von Haeseler, and L. S. Jermiin. 2017. ModelFinder: Fast model selection for accurate phylogenetic estimates. Nat. Methods 14:587–589. https://doi.org/10.1038/nmeth.4285.

Kielland, C., L. Ruud, A. Zanella, and O. Østerås. 2009. Prevalence and risk factors for skin lesions on legs of dairy cattle housed in freestalls in Norway. J. Dairy Sci. 92:5487–5496. https://doi.org/10.3168/jds.2009-2293.

Klaas, I. C., and R. N. Zadoks. 2018. An update on environmental mastitis: Challenging perceptions. Transbound. Emerg. Dis. 65:166–185. https://doi.org/10.1111/tbed.12704.

Li, H., and R. Durbin. 2009. Fast and accurate short read alignment with Burrows–Wheeler transform. Bioinformatics 25:1754–1760. https://doi.org/10.1093/bioinformatics/btp324.

Li, H., B. Handsaker, A. Wysoker, T. Fennell, J. Ruan, N. Homer, G. Marth, G. Abecasis, R. Durbin, and 1000 Genome Project Data Processing Subgroup. 2009. The Sequence Alignment/Map format and SAMtools. Bioinformatics 25:2078–2079. https://doi.org/10.1093/bioinformatics/btp352.

Lundberg, Å. 2015. Mastitis in Dairy Cows: Genotypes, Spread, and Infection Outcome of Three Important Udder Pathogens. Faculty of Veterinary Medicine and Animal Science, Department of Clinical Sciences, and National Veterinary Institute, Department of Animal Health and Antimicrobial Strategies. Doctoral Thesis. Swedish University of Agricultural Sciences, Uppsala.

Lundberg, Å., A. Nyman, A. Aspán, S. Börjesson, H. E. Unnerstad, and K. P. Waller. 2016. Udder infections with *Staphylococcus aureus*, *Streptococcus dysgalactiae*, and *Streptococcus uberis* at calving in dairy herds with suboptimal udder health. J. Dairy Sci. 99:2102–2117. https://doi.org/10.3168/jds.2015-9487.

Lundberg, Å., A. Nyman, H. E. Unnerstad, and K. P. Waller. 2014. Prevalence of bacterial genotypes and outcome of bovine clinical mastitis due to *Streptococcus dysgalactiae* and *Streptococcus uberis*. Acta Vet. Scand. 56:80. https://doi.org/10.1186/s13028-014-0080-0.

McMillan, D. J., D. E. Bessen, M. Pinho, C. Ford, G. S. Hall, J. Melo-Cristino, and M. Ramirez. 2010. Population genetics of *Streptococcus dysgalactiae* subspecies *equisimilis* reveals widely dispersed clones and extensive recombination. PLoS One 5:e11741. https://doi.org/10.1371/journal.pone.0011741.

Mundt, J. O. 1982. The ecology of the streptococci. Microb. Ecol. 8:355–369. https://doi.org/10.1007/BF02010675.

Nguyen, L.-T., H. A. Schmidt, A. Von Haeseler, and B. Q. Minh. 2015. IQ-TREE: A fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol. Biol. Evol. 32:268–274. https://doi.org/10.1093/molbev/msu300.

Norwegian Ministry of Agriculture and Food. 2004. FOR-2004-04-22-665. Regulation on keeping cattle (in Norwegian). Accessed Dec. 21, 2021. https://lovdata.no/dokument/SF/forskrift/2004-04-22-665.

Norwegian Veterinary Institute. 2021a. Ellipsis. https://doi.org/10.5281/zenodo.4563897.

Norwegian Veterinary Institute. 2021b. ALPPACA. https://doi.org/10.5281/zenodo.4452122.

Oliver, S. P., G. M. Pighetti, and R. A. Almeida. 2011. Mastitis pathogens: Environmental pathogens. Pages 415–421 in Encyclopedia of Dairy Sciences, 2nd ed. J. W. Fuquay, ed. Academic Press.

Persson Waller, K., B. Bengtsson, A. Lindberg, A. Nyman, and H. Ericsson Unnerstad. 2009. Incidence of mastitis and bacterial findings at clinical mastitis in Swedish primiparous cows—Influence of breed and stage of lactation. Vet. Microbiol. 134:89–94. https://doi.org/10.1016/j.vetmic.2008.09.004.

Porcellato, D., M. Smistad, S. B. Skeie, H. J. Jørgensen, L. Austbø, and O. Oppegaard. 2021. Whole genome sequencing reveals possible host species adaptation of *Streptococcus dysgalactiae*. Sci. Rep. 11:17350. https://doi.org/10.1038/s41598-021-96710-z.

Quinlan, A. R., and I. M. Hall. 2010. BEDTools: A flexible suite of utilities for comparing genomic features. Bioinformatics 26:841–842. https://doi.org/10.1093/bioinformatics/btq033.

R Core Team. 2021. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. https://www.R-project.org/.

Schukken, Y. H., F. J. Grommers, D. Van de Geer, H. N. Erb, and A. Brand. 1991. Risk factors for clinical mastitis in herds with a low bulk milk somatic cell count. 2. Risk factors for *Escherichia coli* and *Staphylococcus aureus*. J. Dairy Sci. 74:826–832. https://doi.org/10.3168/jds.S0022-0302(91)78231-3.

Seemann, T. 2014. Prokka: Rapid prokaryotic genome annotation. Bioinformatics 30:2068–2069. https://doi.org/10.1093/bioinformatics/btu153.

Shen, W., S. Le, Y. Li, and F. Hu. 2016. SeqKit: A cross-platform and ultrafast toolkit for FASTA/Q file manipulation. PLoS One 11:e0163962. https://doi.org/10.1371/journal.pone.0163962.

Skarbye, A. P., P. T. Thomsen, M. A. Krogh, L. Svennesen, and S. Østergaard. 2020. Effect of automatic cluster flushing on the concentration of *Staphylococcus aureus* in teat cup liners. J. Dairy Sci. 103:5431–5439. https://doi.org/10.3168/jds.2019-17785.

Smistad, M., T. S. Tollersrud, L. Austbø, D. Porcellato, C. Wolff, B. Asal, C. J. Phythian, O. Oppegaard, and H. J. Jørgensen. 2021. Molecular detection and genotype characterization of *Streptococcus dysgalactiae* from sheep flocks with outbreaks of infectious arthritis. Vet. Microbiol. 262:109221. https://doi.org/10.1016/j.vetmic.2021.109221.

Statistics Norway. 2020. Statistics for Agriculture, Forestry, Hunting and Fishing. Accessed Aug. 31, 2021. https://www.ssb.no/en/jord-skog-jakt-og-fiskeri/statistikker/jordhus.

Taponen, S., E. Liski, A.-M. Heikkilä, and S. Pyörälä. 2017. Factors associated with intramammary infection in dairy cows caused by coagulase-negative staphylococci, *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Corynebacterium bovis*, or *Escherichia coli*. J. Dairy Sci. 100:493–503. https://doi.org/10.3168/jds.2016-11465.

TINE. 2020. Årsrapport Kukontrollen og Geitekontrollen [Results from the Norwegian Herd Recording System and Norwegian Goat Recording System]. Accessed Aug. 1, 2021. https://medlem.tine.no/fag-og-forskning/statistikk-2020-for-kukontrollen-og-geitekontrollen/_/attachment/inline/59a4610f-2d7e-4b6f-a37a-7f43c9977306:53e8e6153c6fa6348276b5cc0bc8e9b3a58d6079/Statistikksamling%20husdyrkontrollen%202020.pdf.

Todhunter, D. A., K. L. Smith, and J. S. Hogan. 1995. Environmental streptococcal intramammary infections of the bovine mammary gland. J. Dairy Sci. 78:2366–2374. https://doi.org/10.3168/jds.S0022-0302(95)76864-3.

Tonkin-Hill, G., N. MacAlasdair, C. Ruis, A. Weimann, G. Horesh, J. A. Lees, R. A. Gladstone, S. Lo, C. Beaudoin, R. A. Floto, S. D. W. Frost, J. Corander, S. D. Bentley, and J. Parkhill. 2020. Producing polished prokaryotic pangenomes with the Panaroo pipeline. Genome Biol. 21:180. https://doi.org/10.1186/s13059-020-02090-4.

Treangen, T. J., B. D. Ondov, S. Koren, and A. M. Phillippy. 2014. The Harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes. Genome Biol. 15:524. https://doi.org/10.1186/s13059-014-0524-x.

Vanderhaeghen, W., S. Piepers, F. Leroy, E. Van Coillie, F. Haesebrouck, and S. De Vliegher. 2015. Identification, typing, ecology and epidemiology of coagulase negative staphylococci associated with ruminants. Vet. J. 203:44–51. https://doi.org/10.1016/j.tvjl.2014.11.001.

Wang, S. M., M. A. Deighton, J. A. Capstick, and N. Gerraty. 1999. Epidemiological typing of bovine streptococci by pulsed-field gel electrophoresis. Epidemiol. Infect. 123:317–324. https://doi.org/10.1017/S0950268899002745.

Wente, N., and V. Krömker. 2020. *Streptococcus dysgalactiae*—Contagious or environmental? Animals (Basel) 10:2185. https://doi.org/10.3390/ani10112185.

Whist, A. C., O. Østerås, and L. Sølverød. 2007. *Streptococcus dysgalactiae* isolates at calving and lactation performance within the same lactation. J. Dairy Sci. 90:766–778. https://doi.org/10.3168/jds.S0022-0302(07)71561-8.

Wick, R. R., L. M. Judd, C. L. Gorrie, and K. E. Holt. 2017. Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. PLOS Comput. Biol. 13:e1005595. https://doi.org/10.1371/journal.pcbi.1005595.

Wood, D. E., J. Lu, and B. Langmead. 2019. Improved metagenomic analysis with Kraken 2. Genome Biol. 20:257. https://doi.org/10.1186/s13059-019-1891-0.

Yu, G. 2020. Using ggtree to visualize data on tree-like structures. Curr. Protoc. Bioinformatics 69:e96. https://doi.org/10.1002/cpbi.96.

Yu, G., T. T.-Y. Lam, H. Zhu, and Y. Guan. 2018. Two methods for mapping and visualizing associated data on phylogeny using ggtree. Mol. Biol. Evol. 35:3041–3043. https://doi.org/10.1093/molbev/msy194.

Yu, G., D. K. Smith, H. Zhu, Y. Guan, and T. T. Y. Lam. 2017. ggtree: An R package for visualization and annotation of phylogenetic trees with their covariates and other associated data. Methods Ecol. Evol. 8:28–36. https://doi.org/10.1111/2041-210X.12628.

ORCIDS

M. Smistad https://orcid.org/0000-0002-9282-3594
H. Kaspersen https://orcid.org/0000-0002-9559-1303
C. Wolff https://orcid.org/0000-0002-8661-901X
D. Porcellato https://orcid.org/0000-0002-7460-534X
H. J. Jørgensen https://orcid.org/0000-0002-1788-9219

Supplementary material Paper III

Supplementary Table S1. Results from univariable analyses of herd-level risk factors for having high incidence of *Streptococcus dysgalactiae* subspecies *dysgalactiae* SDSD (case herds) compared to no detected SDSD (control herds), based on data from the Norwegian Herd Recording System and a farmer survey, in total 549 farms. Further multivariable analysis included freestalls only; the results are therefore presented separately for freestalls and tiestalls here.

		Total (n=549)[c]		**Tiestalls (n=184)**		**Freestalls (n=365)**	
Name of variable	**Categories**	**Case (n=132)**	**Control (n=417)**	**Case (n=28)**	**Control (n=156)**	**Case (n=104)**	**Control (n=261)**
Type of milking system (n (%)) [a,*]	Pipeline	28 (21)	156 (37)	28 (100)	156 (100)	n.a.	n.a.
	AMS	70 (53)	222 (53)	n.a.	n.a.	70 (67)	222 (85)
	Parlour	34 (26)	39 (9)	n.a.	n.a.	34 (33)	39 (15)
Herd size (mean, SD) [a,1]		39 (19)	35 (15)	25 (5)	25 (5)	42 (20)	42 (15)
SCC (mean, SD) [a,2]		132 (34)	125 (34)	115 (32)	120 (33)	136 (34)	129 (33)
Annual milk production/cow, mean (SD) [a]		8544 (963)	8450 (1033)	8043 (753)	8146 (1021)	8680 (972)	8632 (999)
Breeding index, mean (range) [a]							
Milk leakage		100.6 (94-106)	100.6 (93-105)	100.9 (95-106)	101 (93-106)	100.7 (94-105)	100.5 (94-105)
Milk flow		99.7 (93-104)	99.7 (93-107)	99.2 (93-104)	99.5 (93-106)	99.9 (94-104)	99.9 (95-107)
Mastitis		99.7 (87-104)	100.0 (94-112)	100.0 (95-104)	99.7 (94-104)	99.7 (87-104)	100.0 (94-106)
SCC		99.3 (92-104	99.7 (92-107)	99.8 (92-104)	99.6 (93-107)	99.0 (94-104)	99.9 (92-106)
Kg concentrate used per 100 kg produced milk (mean (SD)) [a]		31 (5)	31 (5)	31 (4)	31 (4)	31 (5)	31 (5)
Vitamin/mineral supply is given to dry cows (n (%)) [b]	Yes	68 (52)	209 (50)	14 (50)	60 (39)	54 (52)	149 (57)
	No	63 (48)	205 (50)	14 (50)	94 (61)	49 (48)	111 (43)
Type of flooring in alleys (n (%)) [b,*]	Slatted	n.a.	n.a.	n.a.	n.a.	63 (61)	205 (79)
	Closed	n.a.	n.a.	n.a.	n.a.	37 (36)	48 (18)
	Other	n.a.	n.a.	n.a.	n.a.	4 (4)	8 (3)
Building year of the barn (n (%)) [b,*]	Before 2000	132 (100)	417 (100)	28 (100)	156 (100)	28 (26)	44 (17)
	2000-2010	36 (35)	130 (50)	0	0	36 (35)	130 (50)
	After 2010	40 (38	87 (33)	0	0	40 (38)	87 (33)
Type of ventilation (n (%)) [b]	Closed/mechanical	112 (85)	351 (84)	27 (96)	27 (96)	85 (82)	200 (77)
	Open/natural	20 (15)	58 (14)	2 (1)	1 (4)	19 (18)	56 (21)
	Other/combinations	0 (0)	8 (2)	3 (2)	0 (0)	0 (0)	5 (2)
Temperature in the barn (December to February) (n (%)) [b]	0-10°C	63 (48)	193 (47)	1 (4)	41 (27)	62 (60)	152 (59)
	11-15°C	50 (38)	184 (45)	16 (59)	96 (62)	34 (33)	88 (34)
	>15°C	7 (5)	13 (3)	4 (15)	9 (6)	4 (4)	4 (2)
	Adjusted according to outdoor temperature	33 (6)	23 (6)	6 (22)	8 (5)	3 (3)	15 (6)

Observes signs of high humidity in the barn during the winter (n (%)) [b]	Rarely	69 (52)	246 (60)	10 (36)	62 (40)	59 (57)	152 (59)
	Sometimes/often	63 (48)	167 (40)	18 (64)	94 (60)	45 (43)	105 (41)
Type of cubicle base (n (%))*,[b]	Rubber mats	110 (84)	326 (78)	28 (100)	155 (99)	81 (80)	171 (67)
	Mattresses	21 (16)	86 (21)	0	0	21 (20)	86 (33)
Time used for drying off cows (n (%)) [b]	≤ 1 week	69 (53)	223 (54)	14 (50)	82 (53)	55 (53)	141 (55)
	1-2 weeks	52 (40)	161 (40)	13 (46)	68 (44)	39 (38)	93 (36)
	Abrupt	10 (8)	29 (7)	1 (4)	6 (4)	9 (9)	23 (9)
Where the cows are stalled during dry off (n (%)) [b]	In the milking department	58 (56)	135 (52)	n.a.	n.a.	58 (56)	135 (52)
	Calving/VIP pen	16 (15)	34 (13)	n.a.	n.a.	16 (15)	34 (13)
	Dry cow pen	30 (29)	92 (35)	n.a.	n.a.	30 (29)	92 (35)
Type of calving facilities (n (%)) [b]	Calving pen	85 (64)	190 (46)	n.a.	n.a.	85 (82)	190 (73)
	In the milking department	13 (10)	45 (11)	n.a.	n.a.	13 (13)	45 (17)
	Tiestall/outdoors/other	34 (26)	179 (43)	28 (100)	153 (100)	6 (6)	26 (10)
Yearly (or more often) service on the milking machine (n (%)) [b]	Yes	109 (83)	338 (82)	22 (79)	120 (78)	87 (84)	218 (84)
	No	22 (17)	76 (18)	6 (22)	34 (22)	16 (16)	42 (16)
Type of manure scraping (floor) (n (%)) [b]	Manual	27 (26)	78 (30)	n.a.	n.a.	27 (26)	78 (30)
	Robot scraper	35 (34)	120 (46)	n.a.	n.a.	35 (34)	120 (46)
	Mechanical scraper	39 (38)	58 (22)	n.a.	n.a.	39 (38)	58 (22)
	No manure scraping	3 (3)	4 (2)	n.a.	n.a.	3 (3)	4 (2)
Frequency of scraping/rebedding of cubicles (n (%)) [b]	Twice daily	85 (65)	243 (59)	6 (21)	52 (34)	79 (77)	191 (74)
	> Twice daily	41 (31)	142 (35)	22 (79)	99 (64)	19 (18)	43 (17)
	Once daily	4 (4)	26 (6)	0 (0)	3 (2)	5 (5)	23 (9)
Type of bedding material in cubicles (n (%)) [b,*]	Sawdust	121 (92)	365 (88)	28 (100)	145 (93)	93 (89)	220 (84)
	Other	6 (5)	16 (4)	0 (0)	5 (3)	6 (6)	11 (4)
	None	5 (4)	36 (9)	0 (0)	6 (4)	5 (5)	30 (11)
Hygienic focus (free stalls) (n (%)) [b,3]	Low	53 (51)	123 (47)	n.a.	n.a.	53 (51)	123 (47)
	High	51 (49)	138 (53)	n.a.	n.a.	51 (49)	138 (53)
Frequency of cleaning (removing biofilm) from water troughs (n (%)) [b]	Every day	12 (12)	21 (8)	n.a.	n.a.	12 (12)	21 (8)
	1-3 times/week	51 (50)	140 (54)	n.a.	n.a.	51 (50)	140 (54)
	Less often than once a week	40 (39)	100 (38)	n.a.	n.a.	40 (39)	100 (38)
Timing for introduction of heifers to the milking department (n (%)) [b,*]	<3 weeks before calving	34 (33)	73 (29)	n.a.	n.a.	34 (33)	73 (29)
	Week 3-6 before calving	45 (44)	142 (44)	n.a.	n.a.	45 (44)	142 (44)
	>6 weeks before calving	11 (11)	13 (5)	n.a.	n.a.	11 (11)	13 (5)

	After calving	12 (12)	28 (11)	n.a.	n.a.	12 (12)	28 (11)
Calves are fed with waste milk/high SCC milk (n (%)) [b]	Yes/Sometimes	104 (79)	305 (73)	23 (82)	114 (73)	81 (78)	191 (73)
	No	28 (21)	111 (27)	5 (18)	42 (27)	23 (22)	69 (27)
Pregnant heifers are housed with lactating cows (n (%)) [b]	No	94 (90)	232 (90)	n.a.	n.a.	94 (90)	232 (90)
	Yes	10 (10)	25 (10)	n.a.	n.a.	10 (10)	25 (10)
Total		132	417	28	156	104	261

*Univariable screening (freestalls only) $p < 0.2$

[a]Data obtained from the Norwegian Herd Recording system

[b]Data obtained from a farmer survey (questionnaire)

[c]The variables available for both tiestalls and freestalls had a number of missing values ranging from 0-5. Hence the sum does not agree with the total for all variables

[1]Number of lactating cows

[2]Geometric mean bulk somatic cell count, 2018

[3]The variable was based on five statements regarding hygiene in freestalls: routines for clipping of udders, hygiene remarks from slaughterhouse, cleaning of rubber boots before walking in the feed alley, a general focus on keeping cows clean, and perceived problems with keeping the cows clean. E.g. “I always clean my rubber boots when moving from the animal pens to the feed alley” with the possible answers: Always agree-partly agree-disagree. The five statements were combined to one variable by creating a score for each statement (score 1 for low focus, score 2 for high focus). The median total hygiene score was used as a cut-off for low and high hygienic focus.

IV

scientific reports

Check for updates

OPEN

Whole genome sequencing reveals possible host species adaptation of *Streptococcus dysgalactiae*

Davide Porcellato[1], Marit Smistad[2,3], Siv Borghild Skeie[1], Hannah Joan Jørgensen[2], Lars Austbø[2] & Oddvar Oppegaard[4]✉

Streptococcus dysgalactiae **(SD) is an emerging pathogen in human and veterinary medicine, and is associated with several host species, disease phenotypes and virulence mechanisms. SD has traditionally been divided into the subspecies *dysgalactiae* (SDSD) and subsp. *equisimilis* (SDSE), but recent molecular studies have indicated that the phylogenetic relationships are more complex. Moreover, the genetic basis for the niche versatility of SD has not been extensively investigated. To expand the knowledge about virulence factors, phylogenetic relationships and host-adaptation strategies of SD, we analyzed 78 SDSD genomes from cows and sheep, and 78 SDSE genomes from other host species. Sixty SDSD and 40 SDSE genomes were newly sequenced in this study. Phylogenetic analysis supported SDSD as a distinct taxonomic entity, presenting a mean value of the average nucleotide identity of 99%. Bovine and ovine associated SDSD isolates clustered separately on pangenome analysis, but no single gene or genetic region was uniquely associated with host species. In contrast, SDSE isolates were more heterogenous and could be delineated in accordance with host. Although phylogenetic clustering suggestive of cross species transmission was observed, we predominantly detected a host restricted distribution of the SD-lineages. Furthermore, lineage specific virulence factors were detected, several of them located in proximity to hotspots for integration of mobile genetic elements. Our study indicates that SD has evolved to adapt to several different host species and infers a potential role of horizontal genetic transfer in niche specialization.**

Streptococcus dysgalactiae (SD) is a potent pathogen capable of producing a wide spectrum of clinical manifestations and infecting a broad range of host species. Based on DNA-relatedness and phenotypic characteristics, SD is divided into *Streptococcus dysgalactiae* subspecies *dysgalactiae* (SDSD) and subspecies *equisimilis* (SDSE)[1]. SDSD are alpha-haemolytic or non-haemolytic strains belonging to Lancefield group C that are mainly associated with animals, while SDSE are beta-haemolytic strains belonging to Lancefield groups A,C, G or L, and cause miscellaneous infections in humans and domestic animals[1].

SDSD is reported as an important pathogen in meat sheep and dairy cows. The pathogenesis in the two host species is remarkably different. In sheep flocks, SDSD-infections are associated with outbreaks of septic arthritis in lambs less than four weeks old, whilst in bovine dairy herds SDSD is a frequent cause of mastitis.

In Norway, the relative importance of SDSD-infections in livestock has increased over the last decade. In a survey from 2018, 5.6% of 1700 Norwegian sheep farms had experienced outbreaks of infectious arthritis in lambs[2]. At the same time, the prevalence of SDSD intramammary infections is increasing in bovine dairy herds, and SDSD is now the third most common cause of clinical mastitis in dairy cows in Norway[3]. The two industries currently define streptococcal mastitis in dairy cows and streptococcal joint infections in lambs to be among the major challenges in Norwegian livestock production, because of their negative effects on animal health and welfare, production and antibiotic usage.

In the past decades, SDSE has emerged as an important human pathogen. Traditionally, SDSE in humans has been regarded as a potentially zoonotic pathogen, but recent phylogenetic studies based on multilocus sequence typing (MLST) have suggested distinct host-adapted subpopulations of SDSE[4,5].

Although several studies have involved whole genome sequencing of SDSE, very few studies have sequenced SDSD[6,7]. Genomic investigations of SDSD to reveal factors associated with virulence, persistence in the

[1]Faculty of Chemistry, Biotechnology and Food Science, The Norwegian University of Life Sciences, P.O. Box 5003, 1432 Ås, Norway. [2]Norwegian Veterinary Institute, PB 750 Sentrum, 0106 Oslo, Norway. [3]TINE SA, P.O. Box 7, 0901 Oslo, Norway. [4]Department of Medicine, Haukeland University Hospital, 5021 Bergen, Norway. ✉email: Oddvar.oppegaard@helse-bergen.no

environment and host specificity can contribute to enhancing our understanding of pathogenicity and transmission. The purpose of this study was to explore by whole genome sequencing the diversity of SDSD from sheep and cattle in Norway, and to identify genetic factors that might contribute to host adaptation. Furthermore, a comparative genome analysis was performed between bovine- and ovine associated SDSD, and SDSE from various other host species.

Results

Genome statistics of SDSD. In this study, we sequenced 60 new genomes of SD, comprising 37 and 23 isolates from cows and sheep, respectively (Table S1). All the isolates were SDSD with the exception of one ovine isolate which was classified as SDSE. The genomes of an additional 18 SDSD of bovine origin were retrieved from public databases and from Velez et al.[6] and included in the analysis.

The genome size of the 78 SD isolates from cows and sheep had an average of 2.04 MB (2.04 ± 0.1 for bovine isolates and 2.02 ± 0.05 for ovine isolates), an average number of CDS of 1990 (1993 ± 95 in bovine isolates and 1992 ± 40 in ovine isolates).

Virulence factors of SDSD. The genomes of all SDSD isolates of bovine and ovine origin were equipped with numerous virulence genes (Table 1). Several were found to be ubiquitous, including genes involved in adhesion (*fnbA*, *fnbB*, *gapC* and surface enolase), immune evasion (a spyCEP homolog) and dissemination (*padA* and a DNAseB homolog). The adhesin *demA* has previously been characterized in SDSD and was detected in 31 of the 78 SDSD isolates included in this study. Immunoglobulin-binding virulence factors were identified in all genomes, where 48 isolates contained the macroglobulin and immunoglobulin binding protein MIG, and 29 harbored the macroglobulin, albumin and immunoglobulin binding protein MAG.

Notably, pilus-operons were absent from all the SDSD isolates. However, at the genomic location of pilus island 1, they harbored a serine-rich repeat glycoprotein-operon resembling the fibrinogen-binding Srr-locus previously characterized in *Streptococcus agalactiae* (Fig. 1A). In addition to the *srr*-like gene, the operon includes a transcriptional regulator, a SecA2 protein-transport apparatus and three genes putatively involved in Srr-glycosylation, *gtfA*, *gtfB* and *gtfC* (Fig. 1A). Although the sequence homology to the *S. agalactiae* Srr-operon was limited to ~ 50%, the functional domains were conserved.

The most important virulence factor and molecular typing tool of SDSE and *S. pyogenes*, the M-protein, has not previously been identified in SDSD. Interestingly, we located an *emm*-like gene in a genetic context resembling that of the *emm*-gene in SDSE: downstream from *nrdI* and an mgc-regulator, and upstream from 2,3 phosphodiesterase and *relA* (Fig. 1B). The homology is also striking on a protein-level. These M-like proteins have a predicted coiled structure, contain repetitive elements at the C-terminal end, and harbor a YSIRK-signal-peptide and a transmembrane LPxTG anchor with very high homology to SDSE and *S. pyogenes* M-proteins (Fig. 2). The *emm*-typing PCR primers recommended by CDC have 4 mismatches in the forward primer and 1 mismatch in the reverse primer when aligned to the SDSD *emm*-like gene, which likely explains why this subspecies appears to be non-typable using this protocol. Applying the CDC *emm*-typing scheme in silico we categorized the SDSD-genomes into different *emm*-types for phylogenetic purposes (Fig. 3, Table S1). At the recommendation of the curators these new *emm*-like genes have not been deposited in the *emm*-database.

MLST and phylogenetic analysis of SDSD. Molecular typing revealed 14 different MLST-profiles among the SDSD isolates, including 5 novel profiles (Table S1, Fig. 3). Isolates from dairy cows displayed 13 different MLST-types, of which the majority have previously been reported in association with bovine mastitis, while isolates from sheep were more homogenous and grouped into four STs. Two of the twenty-three isolates of ovine origin had an MLST-profile identical to SDSD previously associated with bovine mastitis.

Phylogenetic analysis was reconstructed from 752 gene clusters that were identified as single orthologue genes by the pangenome analysis of all the isolates included in the study. The majority of ovine isolates clustered within 2 main clades (Fig. 3). Pairwise average nucleotide identity was larger than 98% between all the isolates of SDSD from cows, sheep and the human isolate.

Dissection of host specific traits in SDSD. Whole genome comparison of isolates derived from bovine and ovine hosts was performed to identify potential host specific signatures. However, the SDSD genomes were highly homogenous, and no single gene or genetic regions were found to be uniquely associated with host species. Searching for genes displaying < 90% similarity between isolates of bovine and ovine origin we identified several surface exposed virulence factors with high genetic variability. However, these genes also displayed substantial heterogeneity within each host-group, and the allelic variants generally corresponded with the MLST-profile.

Interestingly, the glycosylation gene *gtfC* of the putative *srr*-operon existed in two distinct allelic variants displaying 88% similarity. The distribution of these two variants was highly concordant with origin; 50 of the 55 bovine isolates harbored allele A, while 20 of the 22 ovine SDSD isolates contained allelic variant B (Fig. 3). The ovine isolate identified as SDSE did not harbor an *srr*-operon.

Comparison of SDSD and SDSE. The genomes of 77 SDSE from different host species (human, pig, fish, dog and horse) and one isolate of SDSD of human origin were added to the analysis for comparative purposes (Table S1). These included 40 newly sequenced genomes isolated from human, dog, horse and pig (24, 6, 4 and 6, respectively). In total, 78 SDSD and 78 SDSE were used for phylogenetic analysis (reconstructed from the 752 gene clusters described above). The phylogenetic analysis showed a clear separation of the two subspecies and of isolates from different host species (Fig. 4). In addition, one isolate of human origin clustered with the

	Virulence factor	GenBank ID	SDSD		SDSE				
			Cow ($n=55$) (%)	Sheep ($n=22$) (%)	Human ($n=35$) (%)	Horse ($n=20$) (%)	Dog ($n=8$) (%)	Swine ($n=7$) (%)	Fish ($n=5$) (%)
Adhesin	*fnbA*	Z22150	100	100	0	0	0	0	0
	fnbB	Z22151	100	100	100	100	100	100	100
	demA	AJ243529	38	45	0	0	0	0	0
	pilus1		0	0	100	95	100	100	trunc
	pilus2		0	0	86	25	0	0	0
	gfba / prtf1	U31115	0	0	39	5	100	0	0
	srr	EFY01869	100	100	0	0	0	0	0
	gapC	X97788	100	100	100	100	100	100	100
	Surface enolase	AAT86712	100	100	100	100	100	100	100
	lmb1	AB040535	0	0	100	0	0	0	0
	lmb2	SUN51641	100	100	0	0	0	0	0
	Streptolysin O	AE004092	0	0	100	0	0	0	0
Toxin	NAD	AAK33265	0	0	100	0	0	0	0
	Streptolysin S	AF067649	0	0	100	100	100	100	100
	Sil	KF188416	2	0	64	25	0	100	0
	c5a-peptidase	J05229	0	0	100	0	0	0	0
	spyCEP-like	SUN48961	100	100	trunc	trunc	100	100	100
Immune evasion	proteinG	Y00428	0	0	69	0	0	0	0
	MIG	Z29666	65	55	0	0	0	0	0
	MAG	L27798	35	45	0	0	0	29	0
	drsG	AB508817	0	0	42	0	0	0	0
	DNAseB-like	NP_269989	100	100	0	0	0	0	0
	*mf2**	NP_268944	11	4	0	0	0	0	0
	*mf3**	NP_269520	38	87	8	0	0	0	0
	*mf4**	AAM79702	0	17	0	20	0	0	0
	*sdn**	AAM80016	4	4	8	5	0	57	0
	*sda2**	WP002988811	7	30	17	0	0	0	0
Spread	Streptokinase	K02986	0	0	100	0	0	0	0
	padA	AJ441115	100	100	0	0	0	0	0
	*skc*_horse	AF104301	0	0	0	100	0	0	0
	*skc*_pig	AF104300	0	0	0	0	0	100	0
	Putative skc	WP129556387	0	0	0	0	0	15	100
	Putative skc	VTS33028	0	0	0	0	100	0	0
	*speC**	AAK33664	9	4	0	0	0	0	0
	speG	AF124499	0	0	56	20	100	29	80
Superantigen	*speK**	WP011054728	11	4	0	0	0	0	0
	*speL**	WP011017837	5	0	0	0	0	0	0
	*speM**	WP011017838	5	0	0	0	0	0	0

Table 1. Virulence factors of *Streptococcus dysgalactiae* subsp. *dysgalactiae* (SDSD) and *Streptococcus dysgalactiae* subsp. *equisimilis* (SDSE). Data presented as percentage of isolates harboring the virulence factor. The four isolates that likely represent cross species transmission have been omitted from the table. *Trunc* truncated gene, *skc* streptokinase C. * denotes phage associated genes. No isolates harbored superantigens *speA*, *speH*, *speI*, *speJ*, *speQ*, *speR*, *ssa* or *smeZ*.

SDSD clade. This isolate was obtained from a man suffering from prosthetic valve endocarditis, without verified exposure to livestock[8].

The fastANI algorithm was employed for pairwise comparison of all the genomes. Similar to the phylogenetic analysis, ANI values clearly separated the SD isolates into several clades with distinct delineation of the two subspecies (Fig. 5). The SDSE isolates could be further divided based on the host species. One group contained all the human isolates, except two isolates where the source of infection was suspected to be fish (DB49998-05 and DB60705-15). The second SDSE group contained all the animal isolates, but this group was clearly further separated by host. One isolate from a sheep was identified as SDSE and clustered together with isolates in the pig clade. The average ANI values for the pairwise comparison within the two subsp. were 99.0 and 97.9% for SDSD and SDSE, respectively (Fig. 6). Between the two subsp. the average ANI values were 96.0%. Clear grouping was detected in the ANI value for SDSE, reflecting the two clusters detected by phylogenetic and pangenome analysis.

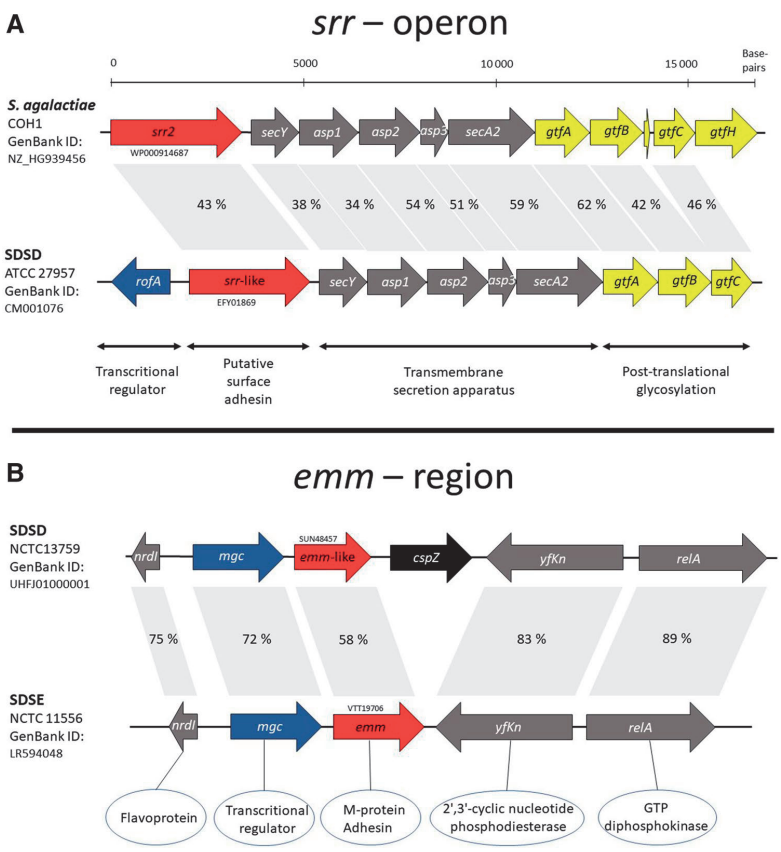


Figure 1. Genetic organization of the Srr-locus and *emm*—region in *Streptococcus dysgalactiae.* Panel A depicts a comparison of the novel *srr*-like locus detected in SDSD to the *srr*-operon of *S. agalactiae*. Two distinct *srr* variants have been described in *S. agalactiae*, denoted *srr1* and *srr2*. The *srr*-like gene and *rofA* transcriptional regulator in SDSD is more similar to *srr1* of *S. agalactiae*. However, the overall genetic organization of the SDSD *srr*-like operon resembles the *srr2*-locus, and is thus presented in the figure. The functional descriptions are inferred from the characterization of the *srr*-locus in *S. agalactiae* (ref Mistou (ref 10)). Panel B shows an alignment of the *emm*—region in SDSD and in human associated SDSE isolates. SDSD genomes harbour an additional gene, *cspZ*, predicted to encode a cell wall surface protein.

Pangenome analysis. Pangenome analysis of the altogether 156 bacterial isolates identified a total of 6464 gene clusters and an estimated pangenome size of 9137 (Chao1 index). Binomial mixture model estimated a pangenome size of 8669 gene clusters and a core-genome of 871 gene clusters (13.5% of total). When considering both subsp. included in the SD species an open pangenomes was detected by Heaps´ law (alpha 0.81). However, clear differences were detected between the two subsp. Isolates of SDSD have a more closed pangenome compared to the SDSE isolates (alpha 0.97 and alpha 0.78, respectively). This also reflects the number of gene clusters identified between the two subsp. (3550 and 5845 for SDSD and SDSE, respectively).

Virulence profiling and host adaptation. All the isolates were screened for presence of virulence factors (Table 1). The adhesins FnbA, DemA and the new putative Srr-glycoprotein were found to be unique for SDSD. Pilus islands and Streptolysin S were restricted to SDSE isolates. Sub specialization within SDSE was observed, and C5a-peptidase, drsG and the toxins streptolysin O and NAD were exclusively detected in human associated SDSE. Moreover, the distribution of various host restricted plasminogen activators (streptokinases) was in concordance with the host lineage they originally were characterized in. Indications of niche adaptation were also evident in genes mediating immune evasion. Human associated SDSE isolates harbored the immunoglobulin binding Protein G, whereas SDSD appears to rely on either MIG or MAG for this purpose.

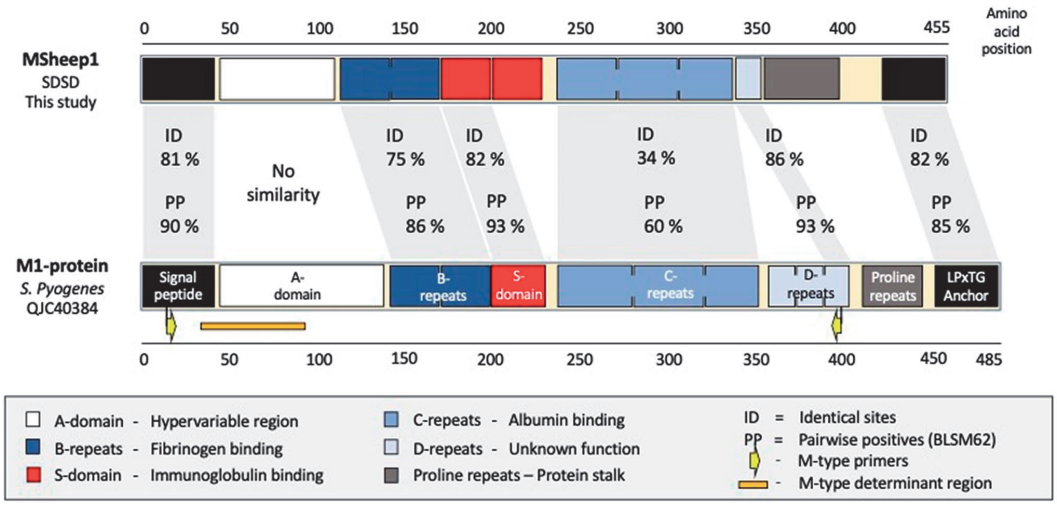


Figure 2. Structure of the M-protein identified in *Streptococcus dysgalactiae* subsp. *dysgalactiae* and in *Streptococcus pyogenes*. Global alignment of the M1-protein of *S. pyogenes* and one of the M-like proteins detected in SDSD in this study. The comparison was performed using Geneious alignment with the BLOSUM62 cost matrix. Sequence similarity is presented as identical sites (ID) and pairwise positives (PP). The proline repeat region functions as a stalk that protrudes the active domains from the bacterial cell surface. It consists of very short proline rich repeats of 3–5 amino acids of variable quantity, making sequence alignment less informative.

To delineate genetic regions potentially mediating host adaptation we performed whole genome comparison of SDSD and SDSE genomes. Due to the limited availability of SDSE isolates of animal origin, we restricted the comparison to human associated SDSE isolates. A total of 17 genetic loci, comprising 40 genes, were found to be unique for and ubiquitously present in SDSD (Table S2). Conversely, 73 genes were specific for human associated SDSE, residing in 19 different genetic regions. The genetic content specific to SDSE displayed high similarity to the strictly human pathogen *S. pyogenes*, whereas genes unique to SDSD resembled virulence factors identified predominantly in animal pathogens (Table S2). Seven of these unique loci harbored well recognized virulence factors, including Streptolysin O, C5a-peptidase and the pilus operons (Figure S1). Moreover, these seven genetic loci were in close proximity to previously characterized hotspots for genetic recombination or insertion of mobile genetic elements.

Mobile genetic elements. Genomes were screened for mobile genetic elements and associated virulence and resistance genes (Table S3). Intact bacteriophages were detected in 81% (63/78) of the SDSD isolates, giving an average of 1.3 bacteriophages per genome (range 0–3). This was a markedly higher prevalence than in human associated SDSE, where 40% (14/35) of the isolates harbored a bacteriophage, average 0.5 per genome (range 0–3) ($p < 0.0001$). This difference between the SD subspecies was also reflected in the carriage of phage-related virulence factors. An average of 1.1 phage-related virulence genes were detected per genome in SDSD versus 0.3 per genome in human SDSE. The streptodornases *mf3* and *sda2* were the most common genes detected in both subspecies, but only SDSD were found to harbor phage-related superantigens, including *speC* (6 isolates), *speK* (7), *speL* (3) and *speM* (3).

Conversely, the prevalence of Integrative Conjugative Elements (ICEs) was significantly higher in SDSE. An average of 2.5 ICEs per genome (range 0–4) were detected in human associated SDSE, compared to 1.6 ICEs in SDSD (range 1–4) ($p < 0.0001$). Carriage of ICE associated resistance genes was generally infrequent but was detected in ten SDSD isolates (2 *tet*(O), 6 *tet*(M) and 4 *lnu*(C), and in six human SDSE isolates (1 *tet*(O), 1 *tet*(M), 1 *mef*(A), and 3 *erm*(A)).

All the SDSD isolates harbored an ICE, Tn5252, equipped with a lactose fermentation operon consisting of 11 genes. Remnants of the ICE and operon was detected in 13 of 35 human SDSE isolates but lacked a conjugation apparatus.

Discussion

To the best of our knowledge, this is the first comprehensive genomic characterization of *Streptococcus dysgalactiae* subspecies *dysgalactiae* (SDSD), and the first study to include isolates of ovine origin. Our findings supported SDSD as a distinct taxonomic entity and revealed several features indicating niche specialization, including the presence of unique virulence factors.

Dissection of the SDSD genomes showed that bovine and ovine isolates formed a tight phylogenetic cluster, displaying a mean value of the ANI of 99% and larger than 98% for all the pairwise comparison. Although the

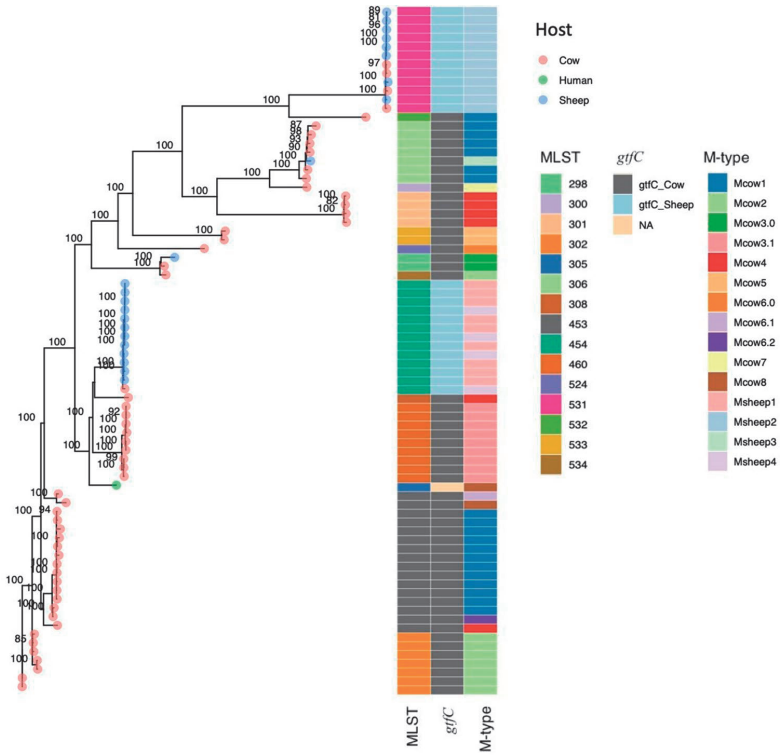


Figure 3. Phylogenetic tree of *Streptococcus dysgalactiae* subsp. *dysgalactiae* isolates obtained from the alignment of 752 single orthologue genes and information about MLST, gtfC-type and M-type. The numbers above the branches are support values from 100 bootstrap replicates.

pangenome analysis divided the SDSD isolates largely in accordance with the animal species from which they were isolated, we did not identify any marker genes specific to host. This short evolutionary distance is surprising in light of the markedly different disease phenotypes this pathogen produces in sheep and cattle. Further exploration of the genome sequences identified one gene, *gtfC,* existing in two distinct allelic variants, and their distribution correlated with host of origin. In *S. agalactiae*, the *gtfC* gene has been verified to encode a glycosylation enzyme involved in post translational modification of the adhesin Srr, leading to modulation of bacterial adherence to host cells[9]. A similar influence on the adhesive properties in SDSD is plausible, and its potential role in host specificity should be further explored.

SD isolates obtained from pigs, dogs, horses, fish and humans were phylogenetically delineated according to source of isolation. The phylogenetic division thus appears to extend beyond the division into the two subspecies, and points to an adaptive evolution of this bacterial species into several host associated lineages. Previous studies based on seven gene MLST have inferred a similar phylogenetic clustering[4,5].

Recently, Nishiki et al.[7] sequenced the first SD isolate from fish, and reported a closer resemblance to SDSD than SDSE. However, their result was influenced by the inclusion *S. equi* in the phylogenetic analysis, reducing the basis of the comparison to 126 core genes. Removing *S. equi* rendered the phylogenetic landscape concordant with our findings, placing fish isolates within the SDSE group. Koh et al.[10] also reported that their fish isolate, STREP97-15, clustered with SDSD when using a seven gene multilocus sequencing analysis. Nevertheless, the STREP97-15 isolate is classified as SDSE based on its reported phenotypic characteristics of beta hemolysis and Lancefield group G antigen. This highlights the complexity in delineating the two subspecies of SD, but also underscores that high phylogenetic resolution should be sought when inferring genomic relationships.

Notably, transmission between different host species appeared to be very rare. Supporting this, Acke et al.[11] did not detect any overlapping MLST-profiles among isolates from cats, dogs and horses, even when these animals had shared the same environment. MLST-types harbored by SDSD-isolates in our study were previously exclusively identified in isolates of bovine origin (MLST-database). However, one isolate obtained from a sheep was identified as SDSE and clustered phylogenetically with the clade of SDSE associated with the porcine host, indicating that the species barrier is not absolute. We have previously published a case of human endocarditis caused by an SDSD-isolate[8], and in the present phylogenetic analysis, this isolate clustered with isolates of bovine

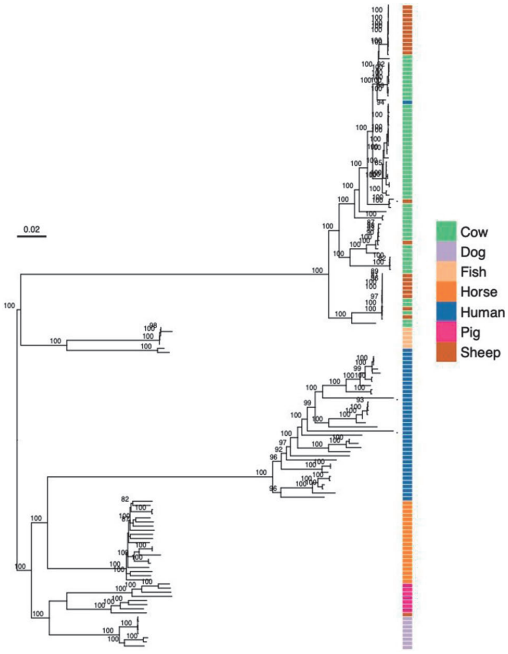


Figure 4. Midpoint-rooted phylogenetic tree of *Streptococcus dysgalactiae* subsp. *dysgalactiae* (n = 78) and *Streptococcus dysgalactiae* subsp. *equisimilis* (n = 78) included in the study. Colours on the side of each plot indicate the origin of the isolate. Scale indicates substitutions per site. The numbers above the branches are support values from 100 bootstrap replicates.

origin. Similarly, a case of a fish handler infected with a presumed piscine SD-isolate has previously been documented in Singapore, and reports of human SD-isolates harboring identical MLST-types as pathogens derived from a pig and a dog has been published in Brazil and Australia, respectively[10,12]. Nevertheless, these case reports appear to represent the exceptions rather than the rule, and zoonotic transmission of this species is likely far less common than previously assumed.

In depth dissection and comparison of the SD genomes presented further indications of niche adaptation, revealing host specific repertoires of virulence factors. SDSD notably lacked the pilus-operons but was equipped with several other tools for adhesion. The fibronectin binding protein FnbA and the fibrinogen binding protein DemA, which were both first described in bovine associated SD-strains, were found to be specific for SDSD in our study[13,14]. In addition, we identified a novel srr/secA2-like operon uniquely present in SDSD strains. Srr has previously been characterized in several streptococcal species, including *S. agalactiae*, and is a heavily glycosylated surface protein mediating adhesion to host tissues[9]. The srr-operon encodes its own apparatus for secretion (secA/Y) and post translational glycosylation. The srr-locus in SDSD comprised all the genes necessary for a functional operon, but the role of this locus in SDSD has yet to be investigated.

In the past decades, several studies have investigated the host specific activity of streptococcal virulence factors. McCoy et al. demonstrated that SD-isolates obtained from horses, pigs and humans were only able to activate plasminogen derived from the homologous host[15]. More recently, the plasminogen activator PadA that is functionally limited to activation of bovine and ovine plasminogen was identified in SDSD[16]. In contrast, human-associated SDSE isolates harbor streptokinase, a close homolog of the plasminogen activator in the strictly human pathogen *S. pyogenes*[16]. We detected host specific streptokinase-like genes in all our SD isolates, although the homologs in dog and fish associated lineages have not been functionally characterized (Table 1).

Not surprisingly, SD appears to have adapted to encounter different host-specific immune systems. The protein MIG detected in SDSD for instance, binds exclusively bovine immunoglobulins[17]. Conversely, the C5a-peptidase of human-associated SDSE, identical to that of *S. pyogenes*, is induced by human serum but not bovine[18]. SDSE isolates of animal origin also appear to harbor host specific genes predicted to have C5a-peptidase and MIG-like activity (data not shown). However, this is based solely on the presence of functional domains, and the properties of these proteins will have to be experimentally verified.

Of particular interest, the majority of the genetic content found to be specific for human associated SDSE displayed high homology to genes harbored by *S. pyogenes* (Table S2). Apart from streptokinase and C5a-peptidase, this included the toxins Streptolysin S, Streptolysin O and NAD-glycohydrolase, as well as the pilus operons and several adhesins. SDSD-specific genes, on the other hand, bore closer resemblance to homologs in other

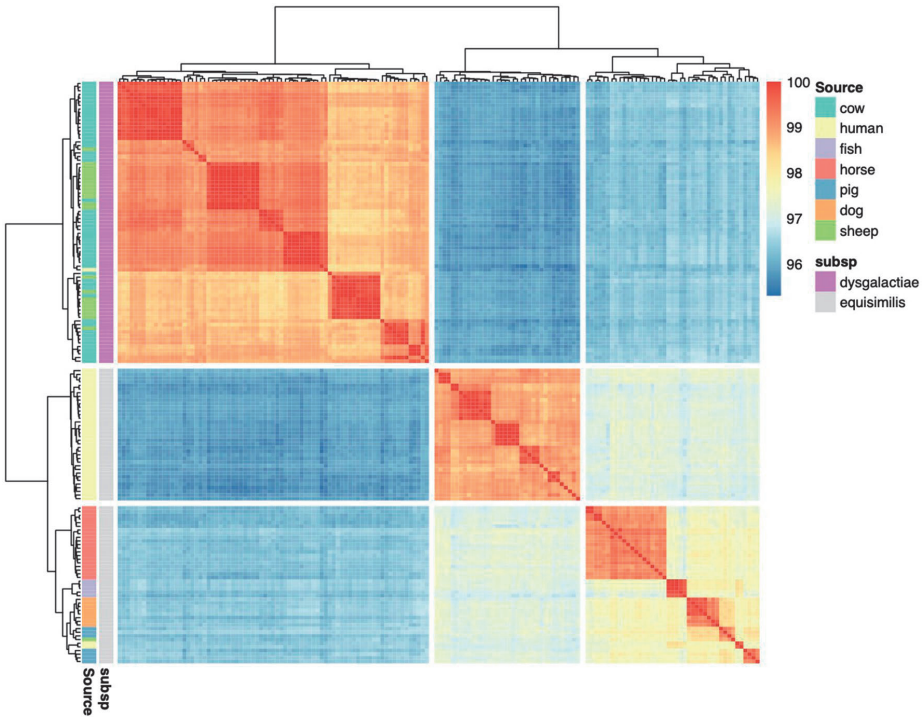


Figure 5. Heatmap of the average nucleotide identity between the whole genome sequences of both subspecies of *Streptococcus dysgalactiae* (n = 156).

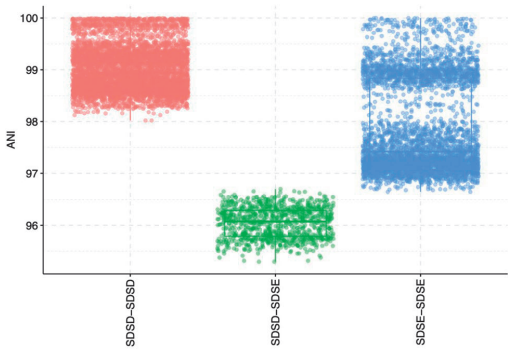


Figure 6. Distribution of pairwise average nucleotide identity within and between the two subspecies of *Streptococcus dysgalactiae*. SDSD: *S. dysgalactiae* subsp. *dysgalactiae,* SDSE: *S. dysgalactiae* subsp. *equisimilis*.

animal associated pathogens, such as bovine *S. agalactiae* (Table S2). It is interesting that with respect to these pivotal loci, the two SD lineages harbor genes with closer resemblance to fellow host pathogens than each other.

In line with this, several of the genetic features delineating SDSD and human associated SDSE are identical to differences previously noted between bovine and human associated *S. agalactiae*, including variable presence of pilus islands and C5a-peptidase (Fig. 7)[19]. Moreover, the acquisition of a novel lactose-fermenting operon (lac2) by bovine mastitis-associated *S. agalactiae* was demonstrated to provide a selective growth advantage in a lactose-rich environment such as milk[20]. The lac2-operon was part of a mobile genetic element, and highly similar elements were detected in other streptococcal species, including one bovine associated SDSD isolate[21]. Interestingly, this lactose-operon and its associated mobile genetic element was found to be ubiquitous in our

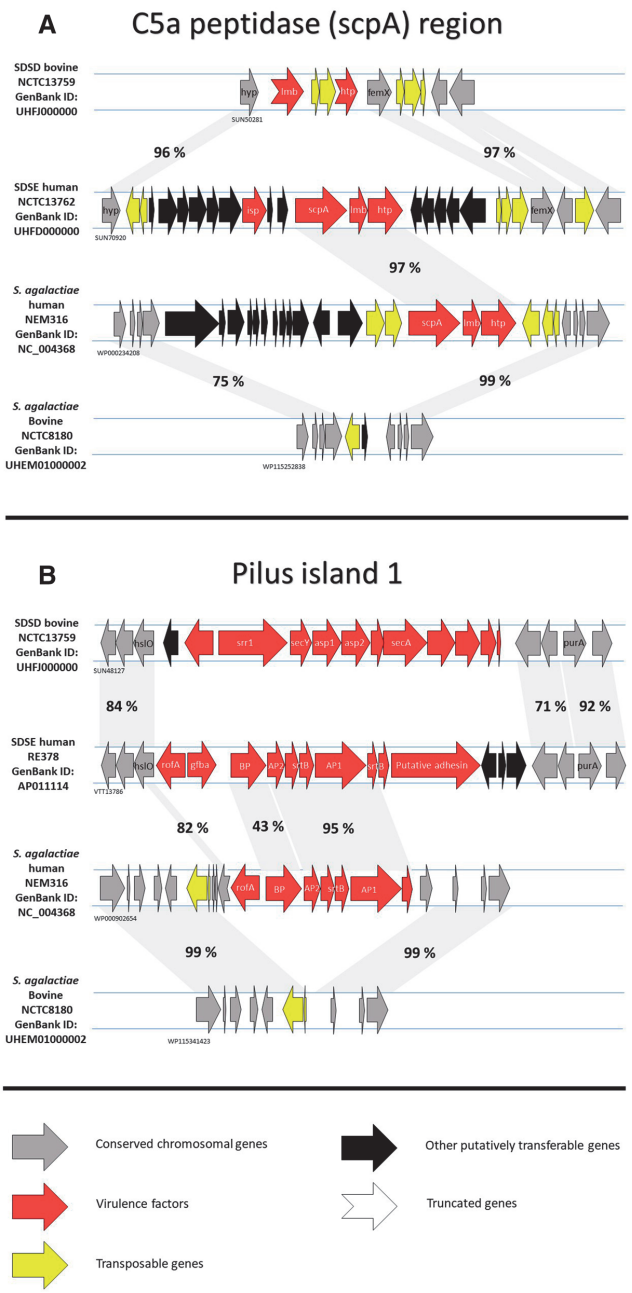


Figure 7. C5a-peptidase and pilus regions in human and bovine SD and *S. agalactiae*. Comparison of genetic features distinguishing bovine and human adapted lineages in *S. dysgalactiae* and *S. agalactiae*. Panel A depicts the C5a-peptidase region, harboring a highly similar *scpA* and *lmb* gene cassette in human associated SDSE and *S. agalactiae*. Pilus regions (Panel B) are absent in bovine SDSD, and bovine *S. agalactiae* are also associated with a lack of the pilus 1 operon. Percentages indicate sequence similarity derived from Geneious alignment. The GenBank protein identity for the first gene in each sequence is indicated.

SDSD-isolates, whereas human associated SDSE isolates only harbored a decayed lac2-element. Taken together these findings suggest a similar adaptive pathway in *S. agalactiae* and SDSD, but also highlight that interspecies horizontal genetic exchange is likely an important strategy for adaptation to new environments.

In sheep, SDSD preferentially targets the joint tissues rather than the udder, and the potential benefits of harboring a lactose operon are less overt. The mobile genetic element might represent an evolutionary remnant in these pathogens. However, they could potentially benefit from increased capability for cross-species transmission, especially in light of the short phylogenetic distance in general between SDSD isolates of ovine and bovine origin.

Environmental genetic transfer as an adaptive strategy has previously been postulated in *S. agalactiae*[22]. SD and *S. agalactiae* have overlapping ecological habitats providing ample opportunity for interaction, and conjugative exchange of mobile genetic elements between these two pathogens has been demonstrated in vitro[23]. Notably, we found several of the loci containing lineage-specific genes to be in close proximity to characterized hotspots for insertion of mobile genetic elements or genetic recombination[24]. In one of these hotspots, we uncovered that all the host specific lineages of SD harbored unique genetic contents, including Streptolysin O in the human lineage, DemA in SDSD, streptokinase in pig isolates and different Protein G-like proteins in canine and piscine associated isolates (Figure S2). Taken together, it seems feasible that the host-specific genome in part represents remnants of cargo genes from past encounters with mobile genetic elements, and that bacteriophages and ICE shape the genetic landscape of SD, contributing to the continuous evolution and niche versatility of these pathogens.

We found SDSD to harbor markedly more bacteriophages than SDSE. This was also reflected in the prevalence of phage-mediated virulence factors such as superantigens and mitogenic factors, in line with a previous array study in these pathogens[25]. In fact, except for the chromosomally encoded *speG*, we could not detect superantigens in any of the human associated SDSE genomes. Bacteriophages are abundant in the farm environment, and interspecies transduction within this milieu could facilitate the high phage-infection rate observed in SDSD[26]. However, the biological implications of being equipped with such armory have yet to be elucidated.

Rosinski-Chupin et al.[27] revealed a reductive evolution to be the most notable in fish-adapted *S. agalactiae* variants, primarily comprising deletion and inactivation of several metabolic functions. In piscine SD isolates, disruption of the *emm*-gene operon and pilus island1 by insertion sequences has been reported[7]. Similarly, we observed insertion sequences affecting other virulence factors in SD, including the deletion of the *emm*-gene in most swine associated SD isolates and the Streptolysin S operon in all SDSD isolates (Figure S2). This suggests that these virulence factors are dispensable in certain host environments and agrees with the notion that a combination of gene loss and acquisition are likely to be involved in niche partitioning[28].

The study is limited by the confined geographic origin of the majority of the SDSD-isolates. However, the phylogenetic clustering and host specific genetic content was conserved also in the genomes procured from public repositories, inferring transferability of our findings to other regions. Nevertheless, future studies involving whole genome sequencing of SDSD-isolates are needed to broaden our understanding of this important pathogen, especially concerning ovine-associated infections. Moreover, characterization of more SD isolates from canine, porcine and piscine sources is warranted to further explore niche specialization and host adaption within this species, and to further refine the taxonomic delineation of SD.

Conclusion

Using whole genome sequencing we reveal that *Streptococcus dysgalactiae* can be delineated into several host specific lineages, and that cross-species transmission appears to be rare. The sublineages are equipped with distinct repertoires of adhesins, toxins and immune evasion proteins likely contributing to host adaption. Moreover, several pivotal genetic loci are in close proximity to hotspots for insertion of mobile genetic elements, suggesting that horizontal genetic transfer could be contributing to niche adaptation and host specificity. The complexity of SD taxonomy is a cause of considerable confusion, and the current subspecies definition could benefit from further scrutiny.

Materials and methods

Bacterial genomes included in the study. A total of 156 genomes sequences, 78 SDSD and 78 SDSE, were analyzed in this study (Table S1). Of the 78 SDSD genomes, 60 isolates were sequenced in the present study and were isolated from dairy cows (n = 37) and sheep (n = 23) in Norway as described below. The other genomes were retrieved from public databases or publications (Table S1). Of the SDSE genomes 40 new isolates were sequenced in this study and the remaining genomes were obtained from public databases. The newly sequenced SDSE genomes included 24 isolates from human and 16 isolates from dog (6), horse (4) and pig (6). Genomes sequenced as part of this study are available at DDBJ/ENA/GenBank under the BioProject PRJEB42928 for the SDSD genomes and BioProject PRJEB43000 for SDSE isolates.

Bovine and ovine SDSD isolates. The bovine and ovine SDSD isolates were collected in a project investigating SDSD diversity in sheep flocks and in bovine dairy herds in Norway (manuscripts in preparation), and the sequence types (ST) of the isolates had already been determined. Ovine isolates were collected between 2016 and 2020 from joint aspirates of lambs with infectious arthritis and body sites of lambs and ewes from 19 sheep flocks. The sheep flocks were located in Northern Norway (n = 14), Western Norway (n = 4) and Eastern Norway (n = 5). One isolate was arbitrarily selected to represent each flock.

Bovine isolates were collected between 2018 and 2020 from quarter milk samples and body sites of cows in eight dairy herds in Eastern Norway. One isolate per ST per herd was arbitrarily selected (range 1–3 STs per herd). In addition, isolates from clinical (n = 10) and subclinical (n = 10) mastitis in dairy cows were randomly selected from the TINE SA mastitis laboratory (Molde, Norway) in the period between March and December 2019. These isolates originated from 20 different dairy herds across the country.

Culturing conditions and DNA extraction. Bacterial isolates were revived and cultured aerobically overnight on blood agar plates with 5% bovine blood (Oxoid). Genomic DNA was extracted using a MagNA Pure 96 instrument (Roche) and MagNA Pure DNA and NA SV Kit (Roche). One µl of bacterial culture was dissolved in 1 ml of phosphate buffered saline, mixed with Bacterial Lysis Buffer 1:1 and mechanically disrupted, 4 times for 1 min, using FastPrep-24 and 2 ml Lysing Matrix B (MP biomedicals). With an input of 200 µl genomic DNA was extracted using the DNA Blood ds SV protocol optimized for double-stranded DNA and NGS and eluted in 50 µl.

Genome sequencing and pangenome analysis. Genomic DNA was quantified using the Qubit 3.0 fluorometer (Life Technologies, Waltham, MA USA). DNA was normalized to 0.2 ng/µL and the sequencing library was prepared using the Nextera XT DNA Sample Prep kit (Illumina, San Diego, California, USA) according to the manufacturer´s instructions. Sequencing was performed using the Illumina MiSeq (Illumina, San Diego, California, USA) and V3 chemistry. Raw sequences were quality filtered using Trimmomatic[29] and de novo assembled using Shovill pipeline (https://github.com/tseemann/shovill). Contigs shorter than 1000 bp and with coverage < 3 were removed prior the annotation step. All the genomes used in this study were annotated using the Prokka pipeline[30]. The protein coding sequences (CDS) were compared with an all-against-all approach, using blastp and the panmatrix was constructed using the R package micropan[31]. CDS were grouped in clusters, using a similarity threshold of 0.75 and complete linkage using the function "bClust" from the micropan package[31]. The R package "micropan" was used to compute openness and closeness of the genomes using Heaps´ law implemented in the function "heaps". The alpha parameter was calculated for all the genomes included in the analysis and for genomes belonging to the two different subspecies of SD. Distances between genomes was calculated from the presence/absence panmatrix by clustering the genomes using Manhattan distances and visualized using the R packages Dendextend[32].

Multilocus sequence typing and phylogenetic analysis. Typing of the isolates was performed using the MLST 2.0 software available at the Center for Genomic Epidemiology webpage (http://www.genomicepidemiology.org/)[33], and novel sequence-types were submitted to the MLST-database (pubmlst.org). The phylogenetic relationship between all SD isolates from the current study (Table S1) was determined using single orthologous genes (defined as genes present in only one copy per genome and obtained from the pangenome analysis). For all gene clusters containing single orthologous genes, present in all genomes, the nucleotide sequences were translated to amino acids, aligned using "Decipher" r-package[34], and back-translated to nucleotide sequences. All alignments were then concatenated into a single file containing all the aligned, single-copy, orthologous genes. Positions with gaps and indels were removed from the final fasta file. A Maximum likelihood tree was constructed using the Geneious software V 10.0.7. with Jukes–Cantor distance, four substitution rate categories and empirically determined gamma substitution parameter with a bootstrap of 100. A second method based on average nucleotide identity (ANI) was performed to compare the genomes using the fastANI algorithm[35]. Clustering of the pairwise comparison of ANI results was constructed using Euclidian distances.

Characterization of virulome, resistome and mobilome. All the genomes were screened for streptococcal virulence factors and resistance genes using Geneious. Bacteriophages were detected using Phaster[36], and Integrative Conjugative Elements (ICE) were identified by a combination of BLAST search and manual inspection of integration hotspots, as previously described[24]. Mann–Whitney U test was used to compare the distribution and quantity of mobile genetic elements in SDSD and SDSE.

In order to locate regions potentially involved in host adaptation, genomes of isolates derived from different host were manually compared and inspected for unique genomic content. The contigs of each individual genome were first sorted by alignment to a reference genome using the MAUVE MCM algorithm[37]. NCTC13759 and NCTC13762 were used as reference for SDSD and SDSE, respectively. The sorted contigs were concatenated, and whole genomes were aligned for comparison using the progressive MAUVE-algorithm. Putatively unique genes and genetic regions were verified through BLAST search against all the genomes. Novel and hypothetical genes were checked for conserved functional domains using the NCBI Conserved Domain BLAST service[38].

Ethical approval. Human isolates were obtained from a study which underwent institutional ethics review and approval (2019/63132 Regional Ethics Committee West, Norway). Farms included in this study operate under the regulations of the Norwegian Food Safety Authority regarding food production and animal care. The farmers provided permission for the sampling and for the use of their information in this study. All methods were carried out in accordance with relevant guidelines and regulations. Invasive samples (joint aspirates) were only collected from sick animals and the sampling was performed by veterinarians in clinical practice as part of the routine diagnostic work, which does not require ethical approval.

Received: 1 March 2021; Accepted: 13 August 2021
Published online: 30 August 2021

References

1. Vieira, V. V. *et al.* Genetic relationships among the different phenotypes of *Streptococcus dysgalactiae* strains. *Int. J. Syst. Bacteriol.* **48 Pt 4**, 1231–1243. https://doi.org/10.1099/00207713-48-4-1231 (1998).
2. Smistad, M. *et al.* Flock-level risk factors for outbreaks of infectious arthritis in lambs, Norway 2018. *Acta Vet. Scand.* https://doi.org/10.1186/s13028-020-00561-z (2020).
3. Østerås, O. HELSEKORTORDNINGEN, STORFE 2018—STATISTIKKSAMLING https://medlem.tine.no/fagprat/husdyrkontrollen/_attachment/499149?_ts=170afd0cd3d (2020).
4. Jensen, A. & Kilian, M. Delineation of *Streptococcus dysgalactiae*, its subspecies, and its clinical and phylogenetic relationship to *Streptococcus pyogenes*. *J. Clin. Microbiol.* **50**, 113–126. https://doi.org/10.1128/JCM.05900-11 (2012).
5. Pinho, M. D. *et al.* Beta-hemolytic *Streptococcus dysgalactiae* strains isolated from horses are a genetically distinct population within the *Streptococcus dysgalactiae* taxon. *Sci. Rep. UK* https://doi.org/10.1038/srep31736 (2016).
6. Velez, J. R. *et al.* Whole-genome sequence analysis of antimicrobial resistance genes in *Streptococcus uberis* and *Streptococcus dysgalactiae* isolates from Canadian dairy herds. *Front. Vet. Sci.* **4**, 63. https://doi.org/10.3389/fvets.2017.00063 (2017).
7. Nishiki, I., Yoshida, T. & Fujiwara, A. Complete genome sequence and characterization of virulence genes in Lancefield group C *Streptococcus dysgalactiae* isolated from farmed amberjack (Seriola dumerili). *Microbiol. Immunol.* **63**, 243–250. https://doi.org/10.1111/1348-0421.12716 (2019).
8. Jordal, S., Glambek, M., Oppegaard, O. & Kittang, B. R. New tricks from an old cow: Infective endocarditis caused by *Streptococcus dysgalactiae* subsp *dysgalactiae*. *J. Clin. Microb.* **53**, 731–734. https://doi.org/10.1128/Jcm.02437-14 (2015).
9. Mistou, M. Y., Dramsi, S., Brega, S., Poyart, C. & Trieu-Cuot, P. Molecular dissection of the secA2 locus of group B *Streptococcus* reveals that glycosylation of the *Srr1* LPXTG protein is required for full virulence. *J. Bacteriol.* **191**, 4195–4206. https://doi.org/10.1128/JB.01673-08 (2009).
10. Koh, T. H., Rahman, N. B. A. & Sessions, O. M. Comparative genomic analysis of *Streptococcus dysgalactiae* subspecies *dysgalactiae*, an occasional cause of zoonotic infection. *Pathology* **52**, 262–266. https://doi.org/10.1016/j.pathol.2019.09.016 (2020).
11. Acke, E. *et al.* Prevalence of *Streptococcus dysgalactiae* subsp. equisimilis and *S. equi* subsp zooepidemicus in a sample of healthy dogs, cats and horses. *N. Z. Vet. J.* **63**, 265–271. https://doi.org/10.1080/00480169.2015.1016133 (2015).
12. Schrieber, L., Towers, R., Muscatello, G. & Speare, R. Transmission of *Streptococcus dysgalactiae* subsp *equisimilis* between child and dog in an aboriginal Australian community. *Zoonoses Public Health* **61**, 145–148. https://doi.org/10.1111/zph.12057 (2014).
13. Lindgren, P. E. *et al.* Two different genes coding for fibronectin-binding proteins from *Streptococcus dysgalactiae*. The complete nucleotide sequences and characterization of the binding domains. *Eur. J. Biochem.* **214**, 819–827. https://doi.org/10.1111/j.1432-1033.1993.tb17985.x (1993).
14. Vasi, J., Frykberg, L., Carlsson, L. E., Lindberg, M. & Guss, B. M-like proteins of *Streptococcus dysgalactiae*. *Infect. Immun.* **68**, 294–302. https://doi.org/10.1128/iai.68.1.294-302.2000 (2000).
15. McCoy, H. E., Broder, C. C. & Lottenberg, R. Streptokinases produced by pathogenic group C streptococci demonstrate species-specific plasminogen activation. *J. Infect. Dis.* **164**, 515–521. https://doi.org/10.1093/infdis/164.3.515 (1991).
16. Ward, P. N., Abu-Median, A. B. A. K. & Leigh, J. A. Structural consideration of the formation of the activation complex between the staphylokinase-like streptococcal plasminogen activator PadA and bovine plasminogen. *J. Mol. Biol.* **381**, 734–747. https://doi.org/10.1016/j.jmb.2008.06.027 (2008).
17. Song, X. M., Perez-Casal, J., Fontaine, M. C. & Potter, A. A. Bovine immunoglobulin A (IgA)-binding activities of the surface-expressed Mig protein of *Streptococcus dysgalactiae*. *Microbiology (Reading)* **148**, 2055–2064. https://doi.org/10.1099/00221287-148-7-2055 (2002).
18. Gleich-Theurer, U. *et al.* Human serum induces streptococcal c5a peptidase expression. *Infect. Immun.* **77**, 3817–3825. https://doi.org/10.1128/IAI.00826-08 (2009).
19. Sorensen, U. B., Poulsen, K., Ghezzo, C., Margarit, I. & Kilian, M. Emergence and global dissemination of host-specific *Streptococcus agalactiae* clones. *MBio* https://doi.org/10.1128/mBio.00178-10 (2010).
20. Richards, V. P., Choi, S. C., Pavinski Bitar, P. D., Gurjar, A. A. & Stanhope, M. J. Transcriptomic and genomic evidence for *Streptococcus agalactiae* adaptation to the bovine environment. *BMC Genomics* **14**, 920. https://doi.org/10.1186/1471-2164-14-920 (2013).
21. Richards, V. P. *et al.* Comparative genomics and the role of lateral gene transfer in the evolution of bovine adapted *Streptococcus agalactiae*. *Infect. Genet. Evol.* **11**, 1263–1275. https://doi.org/10.1016/j.meegid.2011.04.019 (2011).
22. Richards, V. P. *et al.* Population gene introgression and high genome plasticity for the zoonotic pathogen *Streptococcus agalactiae*. *Mol. Biol. Evol.* https://doi.org/10.1093/molbev/msz169 (2019).
23. Haenni, M. *et al.* Diversity and mobility of integrative and conjugative elements in bovine isolates of *Streptococcus agalactiae*, *S. dysgalactiae* subsp *dysgalactiae*, and *S. uberis*. *Appl. Environ. Microbiol.* **76**, 7957–7965. https://doi.org/10.1128/AEM.00805-10 (2010).
24. Ambroset, C. *et al.* New insights into the classification and integration specificity of *Streptococcus* integrative conjugative elements through extensive genome exploration. *Front. Microbiol.* https://doi.org/10.3389/fmicb.2015.01483 (2016).
25. Rato, M. G. *et al.* Virulence gene pool detected in bovine group C *Streptococcus dysgalactiae* subsp *dysgalactiae* isolates by use of a group A *S. pyogenes* virulence microarray. *J. Clin. Microbiol.* **49**, 2470–2479. https://doi.org/10.1128/JCM.00008-11 (2011).
26. Marco, M. B., Moineau, S. & Quiberoni, A. Bacteriophages and dairy fermentations. *Bacteriophage* **2**, 149–158. https://doi.org/10.4161/bact.21868 (2012).
27. Rosinski-Chupin, I. *et al.* Reductive evolution in *Streptococcus agalactiae* and the emergence of a host adapted lineage. *BMC Genomics* **14**, 252. https://doi.org/10.1186/1471-2164-14-252 (2013).
28. Almeida, A. *et al.* Persistence of a dominant bovine lineage of group B *Streptococcus* reveals genomic signatures of host adaptation. *Environ. Microbiol.* **18**, 4216–4229. https://doi.org/10.1111/1462-2920.13550 (2016).
29. Bolger, A. M., Lohse, M. & Usadel, B. Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics* **30**, 2114–2120. https://doi.org/10.1093/bioinformatics/btu170 (2014).
30. Seemann, T. Prokka: Rapid prokaryotic genome annotation. *Bioinformatics* **30**, 2068–2069. https://doi.org/10.1093/bioinformatics/btu153 (2014).
31. Snipen, L. & Liland, K. H. micropan: An R-package for microbial pan-genomics. *BMC Bioinform.* **16**, 79. https://doi.org/10.1186/S12859-015-0517-0 (2015).
32. Galili, T. dendextend: An R package for visualizing, adjusting and comparing trees of hierarchical clustering. *Bioinformatics* **31**, 3718–3720. https://doi.org/10.1093/bioinformatics/btv428 (2015).
33. Larsen, M. V. *et al.* Multilocus sequence typing of total-genome-sequenced bacteria. *J. Clin. Microbiol.* **50**, 1355–1361. https://doi.org/10.1128/Jcm.06094-11 (2012).
34. Wright, E. S. Using DECIPHER v2.0 to analyze big biological sequence data in R. *R J.* **8**, 352–359 (2016).
35. Jain, C., Rodriguez-R, L. M., Phillippy, A. M., Konstantinidis, K. T. & Aluru, S. High throughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries. *Nat. Commun.* **9**, 1. https://doi.org/10.1038/s41467-018-07641-9 (2018).
36. Arndt, D. *et al.* PHASTER: A better, faster version of the PHAST phage search tool. *Nucleic Acids Res.* **44**, W16–W21. https://doi.org/10.1093/nar/gkw387 (2016).
37. Darling, A. E., Mau, B. & Perna, N. T. progressiveMauve: Multiple Geno@me alignment with gene gain, loss and rearrangement. *PLoS ONE* https://doi.org/10.1371/journal.pone.0011147 (2010).

38. Marchler-Bauer, A. *et al.* CDD/SPARCLE: Functional classification of proteins via subfamily domain architectures. *Nucleic Acids Res.* **45**, D200–D203. https://doi.org/10.1093/nar/gkw1129 (2017).

Acknowledgements
The authors would like to acknowledge the Norwegian dairy cooperative TINE SA, Animalia AS, colleagues at the Norwegian Veterinary Institute and the Department of Microbiology at Haukeland University Hospital (Bergen) for their assistance during sampling and analyses. We would also like to acknowledge the microbiological laboratories contributing *S. dysgalactiae* isolates to the NORM surveillance report. This work received financial contributions from the Norwegian Foundation for Research Levy on Agricultural Products and the Norwegian Agricultural Agreement Research Fund (Grant Nos 280364 and 267623), the Western Norway Regional Health Authority (Grant No 912231) and the Norwegian Research council (Grant No. 288917).

Author contributions
D.P.: planning of experiment, performed the experiments and analysed the data, writing of the paper. M.S.: planning of experiment, collection of samples, data analysis and interpretation, writing of the paper, S.B.S.: planning of experiment, writing of the paper. H.J.J.: planning of experiment, writing of the paper. L.A.: sample preparation. O.O.: planning of article, provided data, contributed to data analysis and interpretation, writing of the paper.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-96710-z.

Correspondence and requests for materials should be addressed to O.O.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Supplementary material Paper IV

The online version contains supplementary material (additional file 1-6), available at

https://doi.org/10.1038/s41598-021-96710-z

Figure S1. Pivotal genetic loci distinguishing SDSD and human associated SDSE isolates.

Figure S2. Adaptive evolution in host associated SD lineages.

Table S1. Metadata and genome analysis results for all the strains included in the study, including MLST, M-type and gtfC type.

Table S2. Overview of lineage specific genetic content delineating SDSD and human associated SDSE

Table S3. Mobile genetic elements and associated virulence and resistance genes present in Streptococcus dysgalactiae genomes

Appendix I

Questionnaire - bovine dairy herds (in Norwegian)

Produsentnummer	
Type drift	Båsfjøs, løsdrift uten AMS, løsdrift med AMS
Hvilke raser har du i besetningen?	NRF (>80% av besetningen), Holstein (>80% av besetningen), andre raser eller kombinasjon av ulike raser
Når ble fjøset bygget (oppgi årstall)	
Er fjøset bygget om fra båsfjøs til løsdrift*	Ja/nei
Hva slags ventilasjon har du i melkekuavdelingen?	Mekanisk, naturlig/åpent, annet, vet ikke
Ser du tegn til fuktig miljø i fjøset på vinterstid (des-jan),f.eks damp, drypping fra tak, våte dyr	Nei, sjeldent. Ja, av og til. Ja, ofte. Vet ikke
Hvilken temperatur har du i fjøset på vinterstid (des-februar)	0-5, 6-10, 11-15, >15°C, justeres etter utetemperatur, vet ikke
Hva slags gulv har du i gangarealet (bak liggebåser/ved fôrbrett)*	Spaltegulv, tett gulv, Annet (beskriv)
Hvordan skrapes gulvet?*	Mekaniske skraper, skraperobot, manuelt
Hvor ofte skrapes gulvet?	
Hvor ofte vaskes fjøset ned (gulv, innredning, vegger)?	Hvert år, sjeldnere enn hvert år
Hva slags liggeunderlag har melkekyrne?	Gummimatte, madrass, talle/djupstrø, annet
Hva slags strø brukes der melkekyrne ligger?*	Bruker ikke strø, flis, halm, Drymaxx, Kalk eller hydratkalk, Produs Tørrdes, Stalosan, annet
Hvor ofte brukes spesialstrø*	Hver dag, Annenhver dag, 2-3 ganger per uke, sjeldnere
Hvor ofte skrapes liggebåsene?	En gang daglig, to ganger daglig, oftere enn to ganger daglig (ved behov), annet
Hvor godt stemmer følgende påstander om renhet på melkekyrne - Bakparter og jur klippes ved behov - Jeg får sjeldent hygienetrekk i slakteoppgjøret - Jeg har stort fokus på å holde melkekyrne rene - Jeg har problemer med å holde melkekyrne rene - Jeg begrenser persontrafikk fra løsdrift til forbrett så mye som mulig* - Ved persontrafikk fra løsdrift til forbrett spyles alltid støvlene*	Stemmer godt, stemmer delvis, stemmer dårlig
Hvor ofte rengjøres drikkekar?*	Daglig, 1-3 ganger per uke, ved behov, annet
Fores kalvene med melk fra celletallskyr?	Ja, av og til, nei
Hvor kalver flesteparten av kyrne?*	I kalvingsbinge, i løsdrifta, på bås, i fokusbinge/VIP avdeling, ute, annet
Liggeunderlag for kua i kalvingsbingen*	Liggebås/gummimatter, talle eller djupstrø, annet
Brukes kalvingsbingen til annet enn kalving?*	Nei, ja-syke kyr/kyr under behandling, ja-kyr som skal insemineres, ja-kyr som skal sines av, annet
Hvor oppstalles drektige kviger?	På bås, i egne binger med fullspaltegulv, i egne binger med liggebås/tråkkutgjødsling, i egne binger med talle/djuspstrø, annet
Har du kjøpt inn dyr det siste året?	Nei, ja-innkjøp av kalver, ja-innkjøp av drektige kviger, ja-innkjøp av kyr
Tilvenningstid i løsdrift for kviger	1-2 uker før kalving, 3-6 uker før kalving, de tilvennes etter kalving
Hvor godt stemmer følgende påstander	Stemmer godt, stemmer delvis, stemmer dårlig

- Det tas speneprøver av kyr med høyt celletall (>100.000 i geometrisk midddel) før avsining - Jeg behandler kyr ved avsining der det er anbefalt - Kyr som har høyt celletall over tid, eller ikke responderer på behandling, blir utrangert ved neste anledning	
Har roboten steam*	Ja, nei
Daglig renhold av roboten*	Spyler med høytrykk (varmt vann), spyler med høytrykk (kaldt vann), spyler med lunkent/varmt vann(ikke høytrykk), bruker lunkent/varmt vann og såpe/børste/spyler av, skumlegger og spyler, annet
Hvor lang tid bruker du på avsiningsprosessen?	1 uke eller kortere, 1-2 uker eller lengre, siner av alle brått, annet
Hvor oppstalles kyrne under avsiningsprosessen?*	Kalvingsbinge, sykebinge, Vip/fokus-avdeling, i løsdrifta, sinavdeling, på bås
Hvilke andre tiltak gjøre hos deg for å overvåke og forbedre jurhelsa?	Kukontrollprøver månedlige, jevnlige gjenomganger med veterinær, jevnlige gjennomganger med annen rådgiver, årlig funksjonstest av melkemaskin, årlig service av melkemaskin, fôring: fokus på god vomfunksjon, fôring: fokus på å unngå feite dyr, fôring: sikre god vitamin/mineraldekning i sinperioden, annet

Appendix II

Sampling protocols in sheep flocks and bovine dairy herds

1) Sampling protocol, sheep flocks (lambing season of 2019)

	Sampling site	**n samples requested**
Lambs	Joints	Up to 10
	Ear tag wounds	4
	Navels	4
	Skin	4
	Tonsils/pharynx	4
	Rectum	4
Ewes	Vagina	5
	Nostril	5
	Udder skin	5
	Tonsils/pharynx	5
	Rectum	5
Environment	Floor, interior fittings	3

2) Sampling sheep (fall 2019)

	Sampling site	n samples requested
Lambs (6 month old)	Ear tag lesion	10
	Tonsils/pharynx	10
	Rectum	10
Ewes	Tonsils	10
	Vagina	10
	Rectum	10

3) Sampling bovine dairy herds

Sampling site	n
Milk	10
Body sites	
Wounds	10
Teat skin	10
Skin between the udder and thigh	10
Tonsil calf	5
Vagina	5
Rectum	5
Nostril	5
Environmental sources	
Cubicle bases (four dry and four damp/humid)	8
Calving pen (floor and cubicle base)	2
Milking machine	6-8
Water trough	2-3
Other environmental samples	2-5

ISBN: 978-82-575-2015-1
ISSN: 1894-6402

Norwegian University
of Life Sciences

Postboks 5003
NO-1432 Ås, Norway
+47 67 23 00 00
www.nmbu.no

115683 / ANDVORDGRAFISK.NO