Assessing potential routes of *Streptococcus agalactiae* transmission between dairy herds using national surveillance, animal movement and molecular typing data

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Abstract

Streptococcus agalactiae, also known as group B Streptococcus (GBS), is a pathogen of humans and animals. It is an important cause of mastitis in dairy cattle, causing decreased milk quality and quantity. Denmark is the only country to have implemented a national surveillance and control campaign for GBS in dairy cattle. After a significant decline in the 20th century, prevalence has increased in the 21st century. Using a unique combination of national surveillance, cattle movement and molecular typing data, we tested the hypothesis that transmission mechanisms differ between GBS strains that are almost exclusive to cattle and those that affect humans as well as cattle, which would have implications for control recommendations. Three types of *S. agalactiae,* sequence type (ST) 1, ST23 and ST103 were consistently the most frequent strains among isolates obtained through the national surveillance programme from 2009 to 2011. Herds infected with ST103, which is common in cattle but rarely found in people in Europe, were spatially clustered throughout the study period and across spatial scales. By contrast, strains that are also commonly found in humans, ST1 and ST23, showed no spatial clustering in most or any years of the study, respectively. Introduction of cattle from a positive herd was associated with increased risk of infection by *S. agalactiae* in the next year (risk ratio of 2.9 and 4.7 for 2009–2010 and 2010–2011, respectively). Moreover, mean exposure to infection was significantly higher for newly infected herds and significantly lower for persistently susceptible herds, as compared to random simulated networks with the same properties, which suggests strong association between the cattle movement network and new infections. At strain-level, new infections with ST1 between 2009 and 2010 were significantly associated with cattle movements, while other strains showed only some degree of association. Sharing of veterinary services, which may serve as proxy for local or regional contacts at a range of scales, was not significantly associated with increased risk of introduction of *S. agalactiae* or one of the three predominant strains on a farm. Our findings support the reinstatement of restrictions on cattle movements from *S. agalactiae* positive herds, which came into effect in 2018, but provide insufficient evidence to support strain-specific control recommendations.

Keywords: Mastitis, Multilocus sequence typing, Network analysis, Disease transmission, Spatial clustering

Introduction

Streptococcus agalactiae is an important pathogen of humans, fishes, and dairy cattle. In people, *S. agalactiae*, better known as group B *Streptococcus* (GBS), is a major cause of neonatal infectious disease in the Western world and an emerging invasive pathogen in adults worldwide (Skoff et al., 2009). Most recently, it has been recognized as a cause of foodborne disease in Southeast Asia associated with consumption of raw fish (Barkham et al., 2019). The organism is carried asymptomatically by a large proportion of the adult population, both males and females, primarily in the gastro-intestinal and urogenital tract but also in the oropharynx and on the skin (Bliss et al., 2002; Cobo-Ángel et al., 2019; Van Der Mee-Marquet et al., 2008). In dairy cattle, *S. agalactiae* is known as a cause of intramammary infection, resulting in decreased milk quality and quantity, whence it derives its name. *Streptococcus agalactiae* is often described as an "obligate intramammary pathogen" in the mastitis literature (Mweu et al., 2012a). This characterisation is inaccurate because *S. agalactiae* is also present in other host species, including dogs and cats, and people on dairy farms (Cobo-Ángel et al., 2019; Lämmler et al., 1998; Sørensen et al., 2019). Moreover, it has been detected in bovine faeces in studies in North America (Manning et al., 2010), South America (Cobo-Ángel et al., 2018) and Europe (Jørgensen et al. 2016); in extramammary body sites of cattle such as the throat or vagina (Jørgensen et al. 2016); and in the dairy farm environment (Jørgensen et al. 2016; Cobo-Ángel et al., 2018), showing that mammary, extramammary and environmental sources of *S. agalactiae* may co-exist.

For control of pathogen transmission, it is important to understand the sources and pathways that contribute to dissemination of the organism. In veterinary medicine, the prevailing paradigm guiding GBS control is that it is a contagious pathogen, transmitted from infected individuals to susceptible individuals, whereby the individual can be an animal (within-herd transmission) or a herd (between-herd transmission). Within herds, transmission to susceptible individuals happens as a result of indirect contact during the milking process, e.g., via the milking machine, milkers' hands, or towels used to clean the teats of multiple animals. The risk of transmission can be reduced through use of sanitizer, gloves, and single use towels during milking, as well as post-milking disinfection of teats to kill bacteria left on teat skin (Schukken et al., 2013). In addition, infected cows can be detected and removed through

antimicrobial treatment or culling (Edmondson, 1989; Erskine and Eberhart, 1990; Loeffler et al., 1995; Neave et al., 1969). Antibacterial treatment of GBS mastitis is highly efficacious, with cure reported in more than 96% of affected cows both in lactation (Erskine et al. 1996) and during the dry period (Sol & Melenhorst, 1990; Timone et al., 2018). In Denmark, where selective rather than blanket dry cow treatment with antimicrobials is the standard, the proportion of dry cow treatments is not associated with the likelihood of herd-level recovery from GBS mastitis (Skarbye et al., 2021). Poor control of animal-to-animal transmission within herds leads to greater numbers of infected cows, which increases the risk of transmission between herds, as well as the probability of subsequent animal-to-animal transmission in the recipient herd. In many countries, control of *S. agalactiae* mastitis based on the principles of preventing within- and between-herd transmission has been highly successful (Bauman et al., 2018; Jørgensen et al., 2016; Sampimon et al., 2009).

In Denmark, an initial sharp reduction in herd-level prevalence of *S. agalactiae* from approximately 40% in the beginning of the program in 1960s to 1% in 1989 was followed by an increase in prevalence to almost 5% (Mweu et al., 2012a), and similar trends have been noted in other northern European countries (Jørgensen et al., 2016; Katholm et al., 2012). The gradual increase in prevalence could be associated with a range of causes, including changes in host susceptibility, herd management or pathogen characteristics. Management changes have occurred in association with increasing herd sizes, increased used of automated milking systems, and increased pressure to limit the use of antimicrobials, all of which may contribute to increased opportunities for within-cow and within-herd persistence of *S. agalactiae* infections (Mweu et al., 2012a). Another possible explanation for the limited success of control efforts would be that they are premised on incorrect assumptions with regards to infection epidemiology. Analysis of annual surveillance data for *S. agalactiae* and animal movement records from Denmark showed that the risk of new infection was higher in the period 2005 to 2009 than in the period from 2000 to 2004 but this was not linked to annual movement-related risks, which were the same across both periods (Mweu et al., 2013). This implies that animal movements are not the only route of introduction of *S. agalactiae* infections into previously *S. agalactiae*-free herds and raises the question whether other sources, notably humans, could play a role in introduction of the organism in

susceptible herds. Indeed, there is growing evidence that transmission between people and cattle may occur (Cobo-Ángel et al., 2019; Dogan et al., 2005; Sørensen et al., 2019). A similar phenomenon has been described for *Staphylococcus aureus*, which is also a commensal and pathogen of people and animals, including dairy cattle. For *S. aureus*, both cattle movements and contacts via farm visitors were shown to play a role in its spread between farms (García Álvarez et al., 2011).

Within multi-host pathogens like *S. aureus* and *S. agalactiae*, specialist and generalist strains may exist, i.e., strains adapted to a single host species and those that are commonly found in multiple host species, respectively (Richards et al., 2019; Richardson et al., 2018). For example, studies based on multi-locus sequence typing (MLST) have identified *S. aureus* sequence type (ST)151 and ST425 and *S. agalactiae* ST67 and ST103 as primarily cattle-associated, whereas *S. aureus* ST389 and *S. agalactiae* ST23 are generalist strains that can be found in humans, cattle and other host species (Delannoy et al., 2013; Richardson et al., 2018; Zadoks et al., 2011). It seems reasonable to postulate that transmission patterns differ between specialist and generalist strains, whereby cattle-adapted strains would depend on cattle contacts for transmission whereas generalist strains could be introduced from other sources. Introduction of human strains of *S. agalactiae* into dairy herds may explain its presence in the absence of animal-movement related risks (Dogan et al., 2005; Jensen, 1980). In Denmark, 71% of *S. agalactiae* found in bulk tank milk belonged to ST1, ST23 or ST103 (Zadoks et al., 2011). ST1 and ST23 are host generalists that are also commonly found in people and, in the case of ST23, in a range of terrestrial mammals, aquatic mammals, and cold-blooded species, whereas ST103 is primarily found in bovine milk (Lyhs et al., 2016; Richards et al., 2019; Yang et al., 2013).

The aim of this study was to use the unique combination of annual national surveillance data for *S. agalactiae* and detailed animal movement records as available in Denmark to determine whether spatial distribution and network measures differed between dominant strains of *S. agalactiae*. The underlying biological hypothesis was that the distribution of the specialist strain ST103 would be driven by animal movements, whilst the distribution of generalist strains ST1 and ST23 would be less dependent on animal movements with other plausible routes of transmission being potentially of more importance. Such knowledge could be used to inform *S. agalactiae* control policies in Denmark and other countries.

Materials and methods

Surveillance data

Annual routine surveillance for *S. agalactiae* in the Danish dairy industry has been in place since 1995. Bulk tank milk (BTM) samples from each dairy cattle herd are collected once each year during the period from September to December (inclusive). Samples included in the current study were those from the annual collection periods in 2009, 2010 and 2011 (Additional information S.1; Figure S.1). Some follow-up sampling for 2011 was conducted in 2012, not to determine herd infection status (which was defined by the annual surveillance results) but to obtain bacterial isolates for molecular typing (Additional information S.1; Figure S.2).

During the collection of milk from the bulk tank, the first 30 litres of milk are routinely flushed through the milk hose and pipes in order to avoid false positive test results for *S. agalactiae* due to potential contamination by milk residues from previously visited herds (Andersen et al., 2003). Then, 60 ml of milk was extracted and stored in plastic test tubes that were immediately stored on ice. In the next 24 hours the samples were delivered to Steins Laboratory A/S (Hjaltesvej 8, 7500 Holstebro, Denmark) for examination.

Until the end of 2009, surveillance in Denmark was based on bacteriological culture, using standard methodology for BTM testing (Mweu et al., 2012b). In addition, real-time PCR (PathoProof Mastitis PCR, Finnzymes, Oy, Espoo, Finland) started to be used for BTM screening in 2009. PCR became the standard surveillance method in September 2010. Based on latent class analysis, real-time PCR has higher sensitivity but lower specificity than bacteriological culture, whereby high sensitivity is desirable during an eradication program aimed at detection of every positive herd. However, confirmation by bacteriological culture was considered advisable for herds with high Ct values (Mweu et al., 2012b).

Cases were defined as herds that were GBS positive by PCR, bacterial culture, or both during the annual surveillance period, regardless of the availability of a cultured isolate for molecular characterization. Potential false-positive results due to carry-over between farms (Andersen et al., 2003) were identified using data on the milk collection route and the following criteria: (1) The same ST was identified in the

suspected source herd and the potential recipient of a carry-over event; (2) The pathogen load was lower, i.e. the Ct value was higher, for the suspected recipient than for the suspected source of a carry-over event; and (3) The source herd was persistently infected (GBS positive in multiple years), whereas the recipient herd was not persistently infected (only GBS positive on a single occasion). Herds identified as false positives (n = 14, 6 and 22 for 2009, 2010 and 2011, respectively) were excluded from subsequent analysis (Additional information S.2; Figure S.3).

Attempts to determine MLST profiles using DNA extracted from BTM were not successful (data not shown). Isolates of *S. agalactiae* obtained through bacterial culture were sent to the Moredun Research Institute, where conventional MLST was performed (Jones et al., 2003) or material was prepared for high-throughput MLST (HiMLST) at Streeklab Haarlem, The Netherlands (Boers et al., 2012). Within dairy herds in northern Europe, a single ST of *S. agalactiae* dominates, with occasional occurrence of a single locus variant of the dominant ST. This has been demonstrated in cross-sectional and longitudinal studies in Denmark (Mahmmod et al., 2015), Finland (Lyhs et al., 2016) and Norway (Jørgensen et al., 2016) and is also supported by high resolution typing using whole genome sequencing (Sørensen et al., 2019). Therefore, a single isolate per year was used to determine each herd's ST. Results from the current study confirm this pattern, as the same ST was identified on two or more occasions within a herd for 21 of 22 herds where multiple isolates were evaluated within a single year (Additional information S.3). Likewise, when looking at ST results from consecutive surveillance rounds, results were generally consistent across year. On this basis, missing ST data for PCR-positive, culture-negative BTM samples from annual surveillance rounds were inferred from available ST data from the same herd. In rare cases, assigning STs for confirmed infected herds was not straightforward and the detailed procedures are described in the Supplementary Material (Additional information S.3, Table S.1).

Farm location, animal movement and veterinary practice data

Herd-level data were extracted from the Danish Cattle Database, which allowed us to determine spatial locations of positive and negative herds in 2009, 2010 and 2011, the three years for which molecular typing data were generated. Cattle movement data from 2009 through 2011 were obtained from the

178 Central Herd Register (Danish Veterinary and Food Administration, Glostrup, Denmark) that captures
179 all livestock movements within Denmark on a daily basis. We excluded cattle movements to abattoirs
180 because they were assumed to pose no risk of onward transmission, while movements to and from other
181 agricultural holdings were kept, including movements of young animals that do not produce milk but
182 may carry *S. agalactiae* ((Jørgensen et al., 2016)).

183 We also retrieved a list of registered veterinary practices for each herd and corresponding start and end
184 dates of herd registration with each practice. Data on veterinary practices were used to construct a
185 contact network where a link connects two nodes if the corresponding herds shared the same veterinary
186 practice during the time period of interest. We primarily consider the veterinary contact network as a
187 marker for broader types of professional connections such as sharing of farming equipment, pastures,
188 relief workers, milk or cattle hauliers (García Álvarez et al., 2011), although such connections may exist
189 at a range of spatial scales that differ from those of veterinary contact networks, as shown in Sweden
190 (Olofsson et al., 2014).

191 *Spatial clustering analysis*

192 In order to assess spatial distributions of *S. agalactiae* positive herds and individual STs, we tested for
193 their spatial clustering using Ripley's K function (Ripley, 1981). The function counts the number of
194 cases within a given distance and compares it to the expected number of cases based on a spatially
195 random point pattern. If the number of cases within a given distance of each individual case is greater
196 than that for a random distribution, the distribution is considered clustered. If the number is smaller, the
197 cases are dispersed. Otherwise, if there is no significant difference from the random distribution, the
198 cases are randomly distributed. Ripley's K function is generally calculated at multiple distances to
199 highlight changes in point pattern distributions with spatial scale, e.g., at small distances points could
200 be clustered, while they could be dispersed at a larger scale.

201 The difference between K functions for cases (positive herds regardless of ST or, for ST-specific
202 analysis, herds infected with a particular ST) and controls (all other herds, including *S. agalactiae*
203 negative herds) was used here to account for the heterogeneous underlying population at risk. We also

generated 1000 random ST distributions for fixed herd locations, maintaining the proportion of herds per ST as observed in the dataset. Then we compared the test statistic (difference of K functions) between the observed and simulated ST distributions. Significant clustering of cases is detected if the test statistic for the observed data is higher than for the majority (95% in this case) of randomly generated data. The analysis was performed in R (R Core Team, 2019) using smacpod (French, 2018) and spatstat (Baddeley and Turner, 2005) packages.

Network analysis

The role of particular contacts between dairy herds in pathogen transmission can be assessed using network analysis. In this paper, we considered two types of networks corresponding to cattle movements and shared veterinary services, respectively. The sharing of veterinary services was used as a proxy for other service provider contacts and do not necessarily imply the veterinary visits as the mode of transmission.

Movement network construction

From detailed records of cattle relocation, we derived the number of cows that were in herd A and then appeared in herd B (via direct movement or an intermediate dairy/non-dairy farm, market, etc.) within a pre-defined time period. Dates of first BTM milk samples of national surveillance in each year (19 October 2009, 18 October 2010, and 30 August 2011) determined the two time periods used for the construction of movement networks. The networks for 2009–2010 and 2010–2011 were defined by the following rule: we add an edge to the network if during the considered period there are records that a cow left herd A, entered another herd B, and these events are ordered chronologically. Thus, we capture potential transfer of bacteria from one herd to another, including via cows that could have visited intermediate agricultural premises. The constructed networks assumed that the chance of getting infected with *S. agalactiae* while in transit between herds (e.g., at a market or on communal pasture) is negligible due to a relatively short time and limited number of such movements.

Veterinary network construction

Veterinary networks were based on shared veterinary services, with time periods and nodes defined as described for movement networks. Links connected each pair of herds that shared a veterinary practice at some point during the specified period, thus, these networks were bidirectional as opposed to the movement networks. Also, the constructed veterinary networks were denser than the movement networks, i.e., had more connections on average.

Risk ratio analysis

The risk of becoming infected after having contact with another infected farm can be assessed using the contingency table (Table 1) that summarises possible outcomes for a previously negative farm.

Table 1. Contingency table for risk analysis. We considered the number of newly infected herds (or absence thereof) given absence/presence of incoming links from previously infected herds.

	Event (infection of previously negative farm)	No event (farm stays negative)
Treatment (link from infected farm)	***a***	***b***
Control (no links from infected farms)	***c***	***d***

We calculated the risk ratio (RR) to assess the increase of probability of infection in herds due to an incoming link from an infected herd, i.e., a cattle movement (unidirectional) or the presence of a shared veterinary service (bidirectional). The calculations were performed using the epitools R package (Aragon, 2017). The risk ratios were estimated using unconditional maximum likelihood and the confidence intervals were estimated using a normal approximation. In terms of the contingency table from Table 1,

246 $$RR = \frac{risk\ of\ event\ in\ trt}{risk\ of\ event\ in\ ctrl} = \frac{prob\ of\ infection\ given\ link}{prob\ of\ infection\ with\ no\ link} = \frac{a/(a+b)}{c/(c+d)}$$

247 *Mean exposure analysis*

248 Animal movement and other contact networks, such as those based on shared service providers, have
249 been previously considered in relation to pathogen transmission between dairy cattle herds (García
250 Álvarez et al., 2011; Rossi et al., 2017). We developed a similar approach to compare mean exposure
251 E via links from infectious herds between t_1 (e.g., herd survey in 2009) and t_2 (e.g., herd survey in 2010)
252 for newly infected (NI: negative at t_1 and positive at t_2) and persistently susceptible (PS: negative at
253 both t_1 and t_2) herds:

254 $$E(NI) = \frac{number\ of\ links\ from\ infected\ herds\ at\ t_1\ to\ NI\ herds\ at\ t_2}{number\ of\ NI\ herds\ at\ t_2} = \frac{a}{a+c}$$

255 $$E(PS) = \frac{number\ of\ links\ from\ infected\ herds\ at\ t_1\ to\ PS\ herds\ at\ t_2}{number\ of\ PS\ herds\ at\ t_2} = \frac{b}{b+d}$$

256 In the original paper (García Álvarez et al., 2011), the authors generated randomly simulated networks
257 to assess whether the observed network was significantly different from them in terms of transmission
258 potential. Here, we generated 20,000 random networks by permuted nodes instead, which is effectively
259 equivalent to permuting links and also allowed us to preserve all network properties of simulated
260 networks.

261 To compare results for different years and individual STs, we calculated the proportion P of simulated
262 mean exposure values that were below the mean exposure for the observed data, both for NI and PS
263 herds. We assumed a strong association between pathogen transmission and the network under
264 consideration if $P(NI)$ was higher than 0.95 and $P(PS)$ was lower than 0.05, i.e., observed mean
265 exposure was significantly higher for NI herds and significantly lower for PS herd compared to
266 simulated data. Alternative outcomes including those with $P(NI) > 0.95$ but $P(PS) > 0.05$, and $P(NI) <$
267 0.95 but $P(PS) < 0.05$ were assumed to be insufficient to conclude association of the modelled network
268 structure with transmission.

Results

Molecular characterization of isolates

Population composition of *S. agalactiae* isolated from annual BTM samples was largely consistent between years with three predominant strains ST1, ST23 and ST103 (Figure 1). MLST results were available for 62% (195 of 316), 65% (185 of 284) and 73% (165 of 221) of positive herds in 2009, 2010 and 2011, respectively (with 4258, 4091, and 3918 tested, respectively).

Spatial clustering of infected herds

We tested for spatial clustering of positive herds among all registered dairy herds in Denmark. Our results (Figure 2, upper graphs) suggest significant clustering in 2010, no significant clustering in 2011 and an intermediate result in 2009, when evidence for clustering was only seen at certain spatial scales.

For spatial clustering analysis of individual STs, we present results for the three predominant strains ST1, ST23, ST103. Results for STs that had more than 10 cases over the three years (ST2, ST8, ST9, ST19, ST88 and ST314) can be found in the Supplementary Material (Additional information S.4; Figure S.4). STs that did not have enough cases for the analysis were not examined individually.

Results of K-function analysis (Figure 2, lower graphs) showed herds infected with ST103 were spatially clustered in each year, whereas herds infected with ST23 were not spatially clustered in any year. Herds with ST1, which is the most prevalent ST, showed variable levels of clustering, similar to the overall results for *S. agalactiae* positive herds.

Infection probabilities

In Table 2, we present calculated probabilities of a farm to stay susceptible, become infected, clear infection, etc. The probability of becoming positive was low and decreased from 3.1% in 2009–2010 to 2.1% in 2010–2011. Farms infected with *S. agalactiae* are almost equally likely to recover in the next year (~45%) or stay infected (~50%), with around 4.5% of farms being de-registered from the official database. Probability to stay infected with the same ST (calculated relative to the number of herds infected with that ST rather than to the number of herds that stayed infected with GBS; Table 2) was

59% and 51% in 2009–2010 and 2010–2011, respectively. For major STs, this probability was even higher. Note that we did not account for positive herds with missing ST data, i.e., if a herd was first infected with ST103 and positive in the subsequent year but with no ST assigned, it will be counted as a herd that stayed infected but not with the same ST, so persistence at ST-level may have been underestimated. Infection and recovery rates were similar across years for ST1 and ST23, but differed between years for ST103, with higher risk of becoming infected and higher risk of staying infected in the second year of the study.

Table 2. Infection, recovery, and closure probabilities for Danish dairy herds relative to their *Streptococcus agalactiae* status during annual bulk milk surveillance. We calculated probabilities of changing a status (negative, positive, positive with known ST, positive with missing ST, infected with ST1, ST23, ST103, closure of farm) for 2009–2010 and 2010–2011.

Previous state	**Probability**	**2009–2010**	**2010–2011**
Negative	Stay susceptible	0.92	0.93
Negative	Become infected	0.031	0.021
Negative	Become infected with ST1	0.0025	0.0026
Negative	Become infected with ST23	0.0013	0.0011
Negative	Become infected with ST103	0.001	0.0021
Negative	Closure after being negative	0.046	0.049
Positive	Stay infected	0.51	0.5
Positive	Recover	0.45	0.46
Positive	Closure after being positive	0.041	0.042
Positive with ST	Stay infected with the same ST	0.59	0.51

Any	Closure (removed from the register)	0.045	0.046
ST1	Stay infected with ST1	0.66	0.59
ST1	Recover from infection with ST1	0.24	0.24
ST1	Closure after infection with ST1	0.04	0.061
ST1	Stay infected but with another ST	0.02	0.06
ST1	Stay infected but with unidentified ST	0.04	0.04
ST23	Stay infected with ST23	0.59	0.61
ST23	Recover from infection with ST23	0.23	0.22
ST23	Closure after infection with S23	0.023	0.028
ST23	Stay infected but with another ST	0.09	0.08
ST23	Stay infected but with unidentified ST	0.07	0.06
ST103	Stay infected with ST103	0.49	0.78
ST103	Recover from infection with ST103	0.43	0.087
ST103	Closure after infection with ST103	0.057	0.043
ST103	Stay infected but with another ST	0.03	0.087
ST103	Stay infected but with unidentified ST	0	0

Analysis of cattle movements and shared veterinary services

Risk ratios for *S. agalactiae* infection in Table 3 suggest that cattle movements played a role in the overall pathogen spread in both time periods (risk ratios of 2.9 and 4.7, respectively; significant p-values), while veterinary networks, although significant in 2009–2010, had risk ratios close to one (1.1 and 0.9), i.e., sharing of veterinary services increases risk of infection only marginally.

We also present in Table 3 risk ratios of getting infected with one of the three predominant STs given that there had been a link (cattle movement or shared veterinary service) with a farm infected in the year before. Cattle movements seem to be significantly associated with increased probability of transmission of ST1 in 2009–2010, and of ST23 and ST103 in 2010–2011. For ST103 in 2009–2010, there were no NI herds that bought cattle from herds previously diagnosed with ST103, thus risk ratio could not be calculated. The veterinary network was significant for transmission of ST1 in 2009–2010 and ST23 in 2010–2011, but not for ST103 in either period. Interestingly, absolute values of risk ratios for the movement network were noticeably higher than for the veterinary one.

Table 3. Risk ratios of getting an infection with *Streptococcus agalactiae* (SAG) or its sequence types (ST) in the presence of a link from an infectious farm for movement (MOV) and veterinary (VET) networks.

Network	MOV		VET	
Measure	Risk ratio (CI)	p-value	Risk ratio (CI)	p-value
SAG in 2009–2010	2.9 (1.8-4.7) *	p = 0.00025	1.1 (1.0-1.2) *	p = 0.017
SAG in 2010–2011	4.7 (2.6-8.7) *	p = 0.000086	0.99 (0.89-1.1)	p = 0.83
ST1 in 2009–2010	17 (4.5-66) *	p = 0.0067	1.7 (1.2-2.3) *	p = 0.029
ST1 in 2010–2011	12 (1.7-85)	p = 0.086	1.2 (0.69-2.1)	p = 0.55
ST23 in 2009–2010	11 (1.7-76)	p = 0.089	0.87 (0.41-1.9)	p = 0.72
ST23 in 2010–2011	21 (3.1-150) *	p = 0.05	1.8 (1.2-2.8) *	p = 0.039
ST103 in 2009–2010	No NI herds with links		1.5 (0.66-3.3)	p = 0.43
ST103 in 2010–2011	32 (8.3-130) *	p = 0.002	1.3 (0.7-2.3)	p = 0.47

* Significant association between the observed network and the disease distribution ($p < 0.05$).

Figure 3 shows results of network analysis for the movement and veterinary networks in 2009–2010 and 2010–2011. Potential association between the considered network and pathogen transmission is measured by comparing mean exposure to infection (via direct or indirect links from infectious herds) for NI and PS herds for the observed and random simulated networks. The network is associated with disease spread if mean exposure of NI herds is significantly higher than that of simulated networks (i.e., $P(NI) > 0.95$) and the mean exposure of PS herds is significantly lower ($P(PS) < 0.05$).

The movement networks in both time periods showed significant differences from the simulated networks with higher exposure for NI herds and lower for PS herds compared to random networks. The observed veterinary networks also behaved dissimilarly to the simulated networks, in both time periods mean exposure of NI herds was significantly higher than expected ($P(NI) = 1$), while PS herds also had abnormally higher mean exposure ($P(PS) = 1$). This is suggesting that both NI and PS herds are frequently connected with previously infected ones, which is expected given high density of the veterinary networks. Therefore, we can conclude that the movement networks are associated with increased *S. agalactiae* infection probability but not the veterinary networks.

ST-specific network exposure analysis (Table 4; individual figures can be found in Additional information S.5; Figure S5) showed similar but less pronounced results as those for all *S. agalactiae* types combined. Mean exposure of NI herds via the movement networks was above all simulated networks for all STs and both time periods, with only exception of ST103 in 2009–2010. Although ST-specific analysis generally showed lower mean exposure of PS herds than for simulated networks, infection association criteria for PS herds were met only by ST1 in 2009–2010. The veterinary networks showed extremely high mean exposure of PS herds across STs and time periods. As a consequence, there was no association with ST transmission based on our criteria, not even for STs that also showed higher mean exposure of NI herds.

Table 4. Percentage of exposure to infection with *Streptococcus agalactiae* (SAG) or its sequence types (ST) for simulated random networks that are smaller than the observed exposure for movement (MOV) or veterinary (VET) networks. If the value for NI is above 0.95 and below 0.05 for PS herds, association between the observed network and the disease distribution was significant.

Network	MOV		VET	
Measure	P(NI)	P(PS)	P(NI)	P(PS)
SAG. in 2009–2010	1*	<0.001*	1*	1
SAG in 2010–2011	1*	<0.001*	0.93	1
ST1 in 2009–2010	1*	0.03*	1*	1
ST1 in 2010–2011	1*	0.14	0.88	1
ST23 in 2009–2010	1*	0.25	0.55	1
ST23 in 2010–2011	1*	0.83	1*	1
ST103 in 2009–2010	0.93	0.19	0.91	1
ST103 in 2010–2011	1*	0.16	0.9	1

* Significant association between the observed network and the disease distribution (*P(NI)* > 0.95 or *P(PS)* < 0.05).

Based on both methods, i.e., risk ratios and mean exposure, cattle movements were associated with increased risk of overall *S. agalactiae* transmission in both time periods and were the likely transmission route for new cases of ST1 between 2010 and 2011. Other STs also showed signs of association with cattle movements but did not meet some of our criteria for significance.

While risk ratios of shared veterinary services for all *S. agalactiae* and ST1 in 2009–2010, and ST23 in 2010–2011 were significant, their absolute values were much smaller and closer to one than risk ratios of movements. Moreover, network exposure analysis revealed that veterinary networks increased mean exposure of PS herds to a higher extent than for NI herds, suggesting that shared veterinary practices are unlikely to be associated with infection between herds or that transmission risk is low despite the possibility of such transmission pathways.

Discussion

Making use of Denmark's uniquely detailed data on herd infection status with *S. agalactiae* and animal movements, we show that the risk of herds becoming infected with *S. agalactiae* is significantly associated with movement of animals from positive herds. In addition, we demonstrate strong spatial clustering of ST103 (the third most common ST in Denmark) but not of ST23 (the second most common ST in Denmark), with variable results for ST1 (the most common ST in the country; Zadoks et al., 2011). This analysis extends previous work from Denmark (Mweu et al., 2012a, 2014) in several respects by considering additional years (2009–2011 vs 2000–2009), more detailed movement data, and strain-specific patterns for the three STs that make up ca. 70% of the bovine *S. agalactiae* population in the country. It also differs from prior studies in that herd-level prevalence data up to and including 2009 were based on bacterial culture whereas our data on herd status for 2010 and 2011 were based on PCR. The sensitivity of PCR-based *S. agalactiae* detection in bulk tank milk is considerably higher than for culture, with minimal sacrifice of specificity, whereby point estimates for sensitivity and specificity are 95.2% and 98.8%, respectively, for PCR compared to 68.0% and 99.7% for culture (Mweu et al., 2012b). The incidence of new infections (3.1% of susceptible herds) in 2010 is relatively high compared to the incidence in the preceding years (<2.3%) (Mweu et al., 2012a) and the subsequent year (2.1% in this study), which may reflect the transition from culture-based screening to PCR-based screening with higher sensitivity. Because of the inability to conduct MLST without positive culture results, it is not known whether an association exists between ST and the probability of obtaining culture-negative, PCR-positive results. The lack of ST data could be differential (linked to a specific ST) or non-differential (not ST-specific). Data from a limited number of farms suggests that the average bacterial load in cows infected with ST23 (three farms studied) is lower than for cows infected with ST1 (two farms studied), whereas no data are available for ST103 (Mahmmod et al., 2015). No such results are available at BTM level. The prevalence ratio (within-herd prevalence based on PCR relative to within-herd prevalence based on culture), however, was similar for ST1 and ST23. Thus, culture negative results from PCR positive samples were no more likely for ST23 than for ST1, implying non-differential lack of ST data.

389 Since 2005, there has been an increase in prevalence of *S. agalactiae* positive herds in Denmark (Mweu
390 et al., 2012a, 2014). The infected bovine mammary gland is a major source of the pathogen and often
391 considered the main or even sole driver of transmission of infections. For that reason, there was a
392 prohibition in Denmark to sell cows and pregnant heifers from herds declared to be infected. This ban
393 was lifted in 2005, albeit with the obligation for farmers with infected herds to disclose their herd status
394 (Mweu et al., 2012a). Logically, it was postulated that lifting of the movement restrictions might
395 contribute to the increased herd-level incidence that was subsequently observed. Preliminary analysis
396 of the association between animal movements and herd infection status did not confirm such a link
397 (Mweu et al., 2014), a finding that is contradicted by our analysis. One possible explanation for this
398 discrepancy is the fact that we considered all individual animal movements rather than just the presence
399 or absence of any animal movements. Moreover, infection status of the source herd was taken into
400 account in our analysis, which clearly identified animal movements from infected herds as a risk factor.
401 Thus, our results support reinstatement of movement restrictions of animals from *S. agalactiae* positive
402 herds in the Danish policy. Such measures were effectively implemented in 2018 by registering as
403 infected all milk delivering herds that buy live animals from already registered infected herds.

404 ST103 is among the common STs in areas as far apart as northern Europe (Jørgensen et al., 2016; Lyhs
405 et al., 2016; Zadoks et al., 2011), China (Yang et al., 2013) and Colombia (Cobo-Ángel et al., 2019;
406 Reyes et al., 2017) but it is rarely reported in humans outside Asia. By contrast, ST1 and 23, are
407 common human carriage strains in Europe and North America (Lyhs et al., 2016; Manning et al., 2009).
408 This led us to hypothesize that transmission of ST103, but not ST1 or ST23, would be driven primarily
409 by animal movements, and that different control strategies might be required for different ST in
410 Denmark and other countries where ST103 is prevalent. The concept of strain-specific transmission
411 patterns that inform infection prevention and control measures has relevance well beyond the study
412 setting. For example, methicillin resistant *Staphylococcus aureus* (MRSA) ST398 is 72% less
413 transmissible than other MRSA and does not require the same infection prevention and control measures
414 in hospitals (e.g., patient isolation) as other strains (Wassenberg et al., 2011). Where livestock-
415 associated MRSA ST398 is common compared to other types of MRSA, less stringent control measures

for ST398 MRSA carriers could result in significant cost savings in the health care sector (Bootsma et al., 2011). Conversely, where standard infection prevention and control measures are insufficient to contain spread of a particular strain, additional or alternative control measures may be needed, as illustrated by the emergence of the Delta strain of SARS-CoV-2 (Alizon et al., 2021; Hetemäkiet al., 2021). Although the evidence for movement effect in our study (expressed as risk ratio) was strongest for ST103 (higher effect estimate and lower p-value than ST1 or ST23), it was not convincingly different from estimates for ST1 or ST23. ST103 did differ from ST23, and to a lesser extent from ST1, in that it showed strong spatial clustering. Looking at *S. agalactiae* as a species, evidence of spatial clustering was only observed in one of three years (2010) in the current study, which is consistent with previous observations on lack of spatial clustering at species-level (Mweu et al., 2014). At species-level, the spatial signal from ST103 would be masked by the more common types, ST1 and ST23. *S. agalactiae*, like *S. aureus* and *Streptococcus uberis*, seems to be a more heterogeneous species than previously thought, with different strains showing different transmission patterns (Zadoks et al., 2011). The mechanism underpinning the observed spatial clustering is not clear. There is some suggestion that ST103 may survive in the bovine gastrointestinal tract, with fecal shedding documented in Norway and Colombia, even in cows without *S. agalactiae* mastitis (Cobo-Ángel et al., 2019; Jørgensen et al., 2016). This could potentially indicate an environmental route of transmission for ST103, with manure of carrier animals or farm run-off contributing to spread within or between farms (Jørgensen et al., 2016). Despite the popular misconception that *S. agalactiae* is an obligate intramammary pathogen in the context of dairy farms, the organism can survive in wastewater and aquatic environments for several weeks (Jensen and Berg, 1982), theoretically creating the possibility of local dissemination. All studies that identified ST103 in dairy populations were conducted with isolates collected after 2005, which is due to developments in cost and availability of MLST methodology. Uniquely, archived isolates of *S. agalactiae* are available for a period spanning more than six decades in Sweden. In this collection, ST103 was first detected in 2005, despite testing of 46 isolates obtained from 1952 to 2004 (Crestani et al., 2021). The origin, transmission mechanisms and evolutionary drivers behind the emergence of ST103 are currently unknown. We can however hypothesize that ST103 is a newly emerged strain of *S. agalactiae* with different ecology and transmission than other strains, which may explain its

444 expansion in the face of current control measures in Denmark. Without full understanding of sources
445 and transmission routes, elimination of *S. agalactiae* from the Danish dairy cattle population is likely
446 to remain an elusive goal.

447 There were slight discrepancies in ST-specific results between risk ratios and network exposure
448 analysis. Both methods assess potential association of the considered networks with disease distribution,
449 but they are fundamentally different. Risk ratio indicates differences in frequencies of infections in the
450 presence/absence of infection links for an "average individual", while network exposure analysis aims
451 to compare the observed network with simulated random networks (with the same properties, i.e., that
452 are structurally similar) in terms of mean exposure to infection of NI and PS herds. Therefore, it is
453 possible to observe conflicting results of the two methods.

454 Sharing of veterinary services was not significantly associated with strain-specific and overall spread
455 of *S. agalactiae*. However, the transmission potential via veterinary practices and other between-herd
456 connections needs further analysis, as it could be associated with a variety of other contact types,
457 including but not limited to pasture sharing, haulier routes, or other professional networks (Oloffson et
458 al., 2014). Alternative sources of detailed contact data between dairy herds will be essential to further
459 clarify transmission routes of bovine *S. agalactiae*.

460 **Conclusions**

461 In summary, we demonstrate that strain-level analysis of spatial distributions and contact networks
462 provides enhanced insight into pathogen epidemiology and opportunities for control. Specifically, we
463 demonstrate that the spatial distribution of bovine-associated *S. agalactiae* ST103 is distinct from the
464 spatial distribution of the multi-host strain ST23, and that cattle movements are strongly associated with
465 increased risk of *S. agalactiae* transmission, especially for ST103. This study supports the reinstatement
466 of movement restrictions for live cattle from *S. agalactiae* positive dairy herds in Denmark but provides
467 insufficient evidence to make movement restrictions strain-specific. Further work would be needed to
468 understand the cause of spatial clustering of ST103, and its potential relevance to mastitis control.

Acknowledgements

The authors are grateful to Jørgen Nielsen from the Knowledge Centre for Agriculture in Aarhus, Denmark for providing the herd-level data for the project, and to Ian Heron and Mark Lutton from the Moredun Research Institute for their valuable help with MLST typing.

Funding: MC acknowledges the Lord Kelvin and Adam Smith postgraduate scholarship that supported his Ph.D. at the University of Glasgow. Also, we would like to acknowledge the Danish Milk Levy Fund for their contribution to the costs of MLST.

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645 **Captions for Figures**

646 **Figure 1.** Sequence type (ST) distribution of *Streptococcus agalactiae* isolates collected through annual
647 bulk tank milk surveillance of all dairy herds in Denmark in 2009, 2010 ad 2011. Throughout the study
648 period, ST1, ST23 and ST103 were the most prevalent strains.

649 **Figure 2.** Spatial clustering of herds with *Streptococcus agalactiae* (top row) or its predominant
650 sequence types (ST1, ST23 and ST103; lower rows) among all registered dairy herds in Denmark using
651 K-function and annual bulk tank milk surveillance data from 2009, 2010 and 2011. Clustering is
652 detected when the K-function difference estimate for the observed distribution (red line) is significantly
653 higher than those for randomly generated distributions (shown in light grey, 90% confidence intervals
654 are in dark grey). Light red vertical lines or regions (where line density is high) indicate distances at
655 which spatial clustering is detected.

656 **Figure 3.** Summary of the exposure analysis for the movement ntework (MOV) and veterinary
657 networks (VET) during the year between annual bulk tank surveillance rounds (2009-2010, 2010-2011).
658 Mean exposure to infection (via links from infectious herds) for herds newly infected (NI) with or
659 persistently susceptible (PS) to *Streptococcus agalactiae* is compared between the observed (red dot)
660 and random simulated networks (grey dots). Mean of the simulated values is represented by a black dot.
661 Darker grey regions indicate 0.05 and 0.95 quantiles, and light grey regions indicate ranges of simulated
662 mean exposure values for NI and PS herds. Dashed black line indicates equal mean exposure for NI and
663 PS herds. Proportion *P* of simulated mean exposure values that were below the mean exposure for the
664 observed data are shown on the corresponding axes: NI on x, PS on y.