Herd-related risk factors associated with the severity of clinical mastitis and the incidence of severe mastitis in German dairy herds

*Frederik Fredebeul-Krein[1], Doris Klocke[1], Stefanie Leimbach[1], Julia Nitz[1], Franziska Preine[1], Nicole Wente[1], Yanchao Zhang[1], Volker Krömker[2]**

[1] Hannover University of Applied Sciences and Arts, Faculty II, Microbiology, Heisterbergallee 10a, 30453, Hannover, Germany;
[2] Faculty of Health and Medical Sciences, Department of Veterinary and Animal Sciences, Section for Production, Nutrition and Health, University of Copenhagen, Grønnegårdsvej 2, 1870 Frederiksberg C, Denmark; volker.kroemker@sund.ku.dk
** Corresponding author: Volker Krömker; Phone: +4520693354; E-Mail: volker.kroemker@sund.ku.dk*

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Abstract

Severe mastitis can result in a range of serious general health complications for the infected dairy cow, including septicemia, which can ultimately lead to death. This cross-sectional study aimed firstly to identify the herd-level risk factors associated with severe clinical mastitis (CM) in the diseased dairy cow. The second aim was to investigate herd-related factors associated with the incidence of severe mastitis in the dairy herd. The study was conducted on dairy farms (n = 58) in Northwestern Germany. In addition to data from dairy herd improvement (DHI) tests, possible herd-related predictors were collected on dairy farms. The most frequently isolated pathogens among all CM cases in this study were coliform bacteria (32.6 %), followed by *Streptococcus* (*Str.*) *uberis* (17.2 %). Mastitis cases in clean dairy herds (in > 80.0 % of the examined udders ≤ 10.0 % of the udder surface was soiled), in dairy herds with > two milkings per cow and day, and in herds with a new infection risk (NIR) during the dry period ≤ 28.0 % in the last DHI test prior to CM were identified as herd-related factors associated with more severe CM in the diseased dairy cow. The group of causative pathogens in mastitis cases was another risk factor positively associated with severe CM in the diseased dairy cow. Mastitis caused by coliform pathogens was more likely to be severe than mastitis caused by other pathogens. The mean incidence for severe mastitis in this study was 4.1 severe cases per 100 cow years at risk. The herd milk protein content based on the average of all DHI tests was significantly associated with the incidence of severe mastitis, such that dairy herds with a lower herd milk protein content < 3.4 % were associated with a higher incidence of severe mastitis.

Keywords: bovine mastitis, severity score, risk factors, severe mastitis, cow-years at risk, incidence

Introduction

Mastitis is one of the most prevalent diseases on dairy farms. Research on severe mastitis contributes greatly to improving animal welfare, animal protection, and dairy farm profitability because severe mastitis is directly related to these aspects [1]. To date, there is little evidence of herd-related factors influencing the severity of clinical mastitis (CM) in the diseased dairy cow and herd-related factors influencing the incidence of severe mastitis in the dairy herd. The International Dairy Federation defines severity classification of CM into three mastitis severity scores (MS). MS 1 defines mild mastitis and is characterized by variations in milk texture (color, consistency, viscosity) alone. If there are additional local inflammatory symptoms (swelling, induration, painfulness, redness, increased warmth), the mastitis is classified as moderate mastitis and thus MS 2. Severe mastitis in MS 3 is accompanied by additional systemic clinical signs of illness (fever, hypothermia, permanent recumbency, aversion to feeding) [2, 3, 4]. Severe mastitis can be very consequential for the diseased cow as it can progress to septicemia and even death [5, 6]. In cases of severe mastitis caused by *Escherichia* (*E.*) *coli*, culling rates of 35 % have been described [6]. Locally, irreversible tissue damage often occurs in the diseased glandular quarter [5]. Lower microbiological and clinical cure rates are associated with severe mastitis [7, 8]. In addition, there is economic damage from severe mastitis due to animal losses, production losses, and veterinary costs [9, 10]. Finally, the prognosis for the regeneration of the diseased glandular quarter and the survival of the diseased cow decreases as the severity of mastitis increases [5, 6]. The distribution of mild, moderate, and severe mastitis cases in previous studies ranged from 36-55 % for mild, 36-43 % for moderate, and 9-21 % for severe cases [2, 3, 11].

Previous studies showed that the majority of severe mastitis cases were caused by environment-associated pathogens, especially coliform pathogens (42.2 % of severe CM cases) [2, 3, 5]. Some studies found that mastitis caused by *E. coli* was more severe than mastitis caused by other pathogens [11, 12, 13]. The term "coli mastitis" is often used by veterinarians and farmers as a synonym for severe mastitis [14]. However, Schmenger *et al.* (2020) showed that severe mastitis can be equally caused by Gram-positive microorganisms such as *Streptococcus* (*Str.*) *uberis* [3]. In a previous study, we found that the interaction of the pathogen group and its increased pathogen shedding, especially for coliform pathogens, was associated with the severity of CM [2]. Moderate and severe mastitis were significantly more common in herds with low bulk milk somatic cell counts (BMSCC)

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than in herds with high BMSCC [13]. Dairy cows with severe mastitis had a higher daily milk yield than those with mild mastitis in the last dairy herd improvement (DHI) test before the onset of mastitis [11].

The transition period of a dairy cow poses great challenges for the metabolism and the immune system. Early lactation of the dairy cow was associated with severe courses of mastitis [2, 14, 15]. CM caused by coliform pathogens such as *E. coli* was more severe in early lactation [13, 16]. After peak lactation, mastitis caused by coliform pathogens was often mild and self-limiting [14]. One challenge of the dairy cow in the peri- and postpartum period is low dry matter intake coupled with increased nutrient requirements due to the increase in milk yield and uterine regression compared to other lactation stages [14, 17]. For this reason, a dairy cow's metabolism almost always enters a negative energy balance (NEB) in early lactation. The extent of NEB depends on the extent of the inflammatory reactions and the concomitant immune-induced hypophagia [18, 19]. Dairy cows in an NEB have a reduced function of polymorphonuclear neutrophil granulocytes (PMN) and thus have a suppressed immune system [14, 17, 20, 21]. NEB was associated with severe mastitis [21]. In early lactating dairy cows, NEB was negatively correlated with plasma antibody concentration and SCC in milk [22]. Some studies showed an association between higher parity and severe courses of CM [14, 15], but other studies did not [2, 11]. Primiparous dairy cows have a higher PMN viability and higher neutrophil reactive oxygen species production than multiparous dairy cows [14, 15].Exposure to environment-associated pathogens such as *E. coli* was increased by overcrowding, which often led to excessive contamination [14, 23]. Dairy herds with dirty udders had an increased incidence of CM and of CM caused by *E. coli* [12, 23, 24, 25]. The incidence and the severity of CM could be reduced by keeping a herd clean and comfortable with calm handling [26]. Moderate and severe mastitis were significantly more common in herds that were kept indoors throughout the year than in herds that had access to pasture [13]. CM caused by *E. coli* had more severe courses during the housing period [13]. Separate areas for diseased cows, nightly pasture, and barn areas with slatted floors were associated with a lower incidence of CM caused by *E. coli* during the housing period [23].

Heat stress is defined as the interaction of temperature, humidity, solar radiation, air motion, and precipitation that affect the animal welfare and productivity of dairy cows [27]. Especially during the summer months, insufficient air conditioning of the dairy cow's environment can lead to immune suppression due to heat stress [28]. Heat stress increases SCC in milk and decreases immunoglobulin G, inflammatory cytokines, and the amount and function of PMN [29, 30]. Higher ambient temperatures are associated with higher pathogen loads due to favorable living and multiplication conditions for many pathogens [28]. Smith *et al.* (1985) reported favorable growth conditions for coliform pathogens in bedding due to high temperatures and humidity [16]. Acute coliform mastitis occurred much more frequently during rainy summer months [16]. There were associations between the number of coliform pathogens in the bedding material, the pathogen load at the teat end, and the incidence of CM due to coliform pathogens [30]. Vaccinations against mastitis-associated pathogens such as *E. coli* and *Staphylococcus* (*S.*) *aureus* were associated with milder courses of mastitis, but inhibition of bacterial growth by elevated immunoglobulin G was limited [31, 32, 33].

To date, there is a lack of knowledge about herd-related factors influencing the severity of CM in the diseased dairy cow and the incidence of severe mastitis in the dairy herd. What makes mastitis severe? Are there checkpoints at herd-level to mitigate or avoid severe courses of mastitis? Can herd-specific risk factors for the severity of CM be deduced from already known animal-specific risk factors? Why do relatively more cases of severe mastitis occur in some dairy herds than in others? The aim of this present cross-sectional field study was, on the one hand, to survey herd-related factors associated with severe CM in the diseased dairy cow. On the other hand, another objective was to investigate herd-related factors associated with the incidence of severe mastitis in the dairy herd. Knowledge of these risk factors would allow better and earlier identification of those farms at increased risk for outbreaks of severe mastitis and would provide a first basis to perform further analyses in a risk herd on an animal-specific level. Knowledge of the risk factors may provide the basis for randomized controlled trials to investigate the precise influence of these factors on the severity of CM in the diseased dairy cow and on the incidence of severe mastitis in the dairy herd.

Materials and Methods

Herds and study design: The cross-sectional study was conducted on 58 dairy farms in Northwestern Germany, located in North Rhine-Westphalia (NRW) and Hesse, in the period between June 2020 and September 2020. This study's dairy farms were largely selected from the clientele of the veterinary practice Tierärztliche Gemeinschaftspraxis Büren FGS-GmbH in Büren, Germany, and further supplemented by additional motivated dairy farms from the surrounding region. To participate in the study, farms were required to conduct a monthly DHI test. In the study, data were analyzed from the last DHI test before the onset of CM from participating farms with herd sizes of 35-390 dairy cows of Holstein Friesian and Brown Swiss breeds, with an average rolling 305-day milk yield of 6,000-12,700 kg and average BMSCC of 100-470 kcells per milliliter (mL). According to a 2020 annual report from the North-Rhine Westphalia State Control Association, the average farm size in South Westphalia was 87.5 cows per farm with an average rolling 305-day milk yield of 9,543 kg and a herd average SCC of 214 kcells per mL based on DHI tests [34]. The risk factors (independent variables) in this study were selected based on literature (Table 1). In addition, the independent variables were supplemented by a variety of information from the DHI test and by practical veterinary experience.

Sampling: In the study, milk samples were collected from udder quarters with CM by farm managers and veterinarians in test tubes containing the preserving agent boric acid (Ly20) in a four-month period from June 2020 to September 2020 [35]. The milk samples were immediately sent to the laboratory at the Hannover University of Applied Sciences and Arts, Hannover, Germany, for cytomicrobiological examination. Farm managers and veterinarians were trained in aseptic collection of milk samples according to German Veterinary Association guidelines [36] and in classifying the severity of CM using International Dairy Federation definitions [4]. All farm managers and veterinarians were instructed in evidence-based mastitis therapy before the start of the study. Thus, severe mastitis should always be treated immediately after sampling with antiphlogistic therapy with a nonsteroidal anti-inflammatory drug (NSAID), appropriate parenteral antibiotics, and fluid therapy [37]. At best, the use of local antibiotics should be contingent on the results of a rapid test [37]. However, as the treatment of mastitis is not relevant to the topic and results of this study, this was not further reviewed and evaluated during the study.

Protocol: The farm manager or veterinarian recorded in a protocol the identification of the diseased dairy cow, the day of onset of CM, and the classification of the severity of CM according to the International Dairy Federation definitions [4]. Furthermore, a questionnaire was

Table 1: Definitions of independent variables

Independent Variable	Definitions	Categories	References
Udder cleanliness	1: no soiling on the udder surface (< 2.0 % of the udder surface) 2: mild soiling (2.0 to 10.0 % of the udder surface) 3: moderate soiling (10.1 to 30.0 % of the udder surface) 4: severe soiling (> 30.0 % of the udder surface)	**Clean herd**: > 80.0 % of the cows = Score 1 & 2 **Dirty herd**: ≤ 80.0 % of the cows = Score 1 & 2	12, 23, 26, 27, 28, 38, 39
Number of milkings	The average number of milkings per dairy cow and day in a herd.	2 milkings / cow / day > 2 milkings / cow / day	44, 45
NIR[1] dry period[2]	Number of cows ≤ 100 kcells per mL[3] in the last DHI[4] test before the dry period and > 100 kcells per mL in the first DHI test after the dry period / number of cows ≤ 100 kcells per mL in the last DHI test before the dry period.	NIR ≤ 28.0 % NIR > 28.0 % No value	42, DHI
Pasturing	The dairy cows have access to pasture for at least 120 days a year.	Pasturing; No pasturing	13, 23
Feed hygiene[5]	No reheating and no obvious microbiological spoilage in the lactating ration or in components of the lactating ration in the last 14 days before the case.	Feed hygiene No feed hygiene	46, 47
Air conditioning	The dairy barn has area ventilation and cooling for the lactating cows by active air movement and/or water cooling.	No air conditioning Air conditioning	16, 23, 29, 30, 48, 49, 50, 51, 52
Stocking density[5]	Overcrowding in the dairy barn of lactating cows occurs when a cow number-to-lying space ratio of 1:1 and/or a cow number-to-feeding space ratio of 1:1 is not maintained.	No overcrowding Overcrowding	14, 23
Average herd milk yield / cow / day[2]	The average herd milk yield per cow per day in the last DHI test before the case.	< 33 kg[6]/ cow / day ≥ 33 kg / cow / day	11
Average herd milk fat content / cow[2]	The average herd milk fat content per cow in the last DHI test before the case.	< 4.0 % ≥ 4.0 %	23, 25, 53, DHI
Average herd milk protein content / cow[2]	The average herd milk protein content per cow in the last DHI test before the case.	< 3.4 % ≥ 3.4 %	23, 25, 53, DHI
Average herd milk urea content / cow[2]	The average herd milk urea content per cow in the last DHI test before the case.	< 170 ppm[7] ≥ 170 ppm	53, DHI
Average herd SCC[8] per mL / cow[2]	The average herd SCC per mL per cow in the last DHI test before the case.	< 200,000 SCC per mL / cow ≥ 200,000 SCC per mL / cow	13, 23, 51, 52, 54, 55
Proportion of udder healthy cows[2]	Number of cows ≤ 100 kcells per mL in the last DHI test before the case / number of all cows in the last DHI test before the case.	< 50.0 % ≥ 50.0 %	13, 23, 42, 51, 52, 54, 55, DHI
NIR lactation[2]	Number of cows ≤ 100 kcells per mL in the last DHI test and > 100 kcells per mL in the following DHI test / number of cows ≤ 100 kcells per mL in the last DHI test.	NIR ≤ 21.0 % NIR > 21.0 %	42, DHI
Heifer mastitis rate[2]	Number of heifers ≤ 100 kcells per mL in the first DHI test of their lactation / number of all heifers in the DHI test.	≤ 40.0 % > 40.0 %	42, DHI
Average milking days / cow[2]	The average milking days per cow in the last DHI test before the case.	< 190 ≥ 190	2, 14, 15, DHI
Total starch content in the lactating ration[5]	The calculated total starch content in the lactating ration in the last 14 days before the case.	≤ 20.0 %; 20.0 % No value	23, 53
Feed changes[5]	The feed changes in the lactating ration in the last 14 days before the case.	No feed changes One or more feed changes	-
Total number of cows[2]	The total number of cows in the last DHI test before the case.	< 140 ≥ 140	DHI
Number of milking cows[2]	The number of milking cows in the last DHI test before the case.	< 120 ≥ 120	DHI
Proportion of heifers in the herd[2]	Number of first lactation cows in the last DHI test before the case / total number of cows in the last DHI test before the case.	< 33.0 % ≥ 33.0 %	14, 15, DHI
Average rolling 305-day milk yield / cow[9]	The average rolling 305-day milk yield per cow in the last DHI test control year with 11 DHI tests.	< 9543 kg / cow ≥ 9543 kg / cow	11, 36
Average annual herd SCC per mL / cow[9]	The average annual herd SCC per mL per cow in the last DHI test control year with 11 DHI tests.	< 200,000 SCC per mL / cow ≥ 200,000 SCC per mL / cow	13, 23, 51, 52, 54, 55
SCC per mL of the diseased cow[2]	The individual SCC per mL of the diseased cow in the last DHI test before the case.	< 100,000 SCC per mL / cow ≥ 100,000 SCC per mL / cow	13, 23, 51, 52, 54, 55

[1]new infection risk, [2]in the last dairy herd improvement test before the case, [3]milliliter, [4]dairy herd improvement, [5]in the last 14 days before the case, [6]kilogram, [7]parts per million, [8]somatic cell count, [9]in the last DHI test control year.

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used to collect animal-related data and health parameters at the time of sampling.

Visit of the participating dairy farms: Each participating dairy farm was visited once between June 2020 and September 2020 for the collection of possible herd-related predictors of severe CM (Table 1). The udder hygiene score was used to determine udder cleanliness [38]. For this purpose, at least 25.0 %, usually about 50.0 %, of each herd was classified. Udder cleanliness was differentiated into four scores (Table 1). Finally, in more detailed evaluations, we combined scores one and two (≤ 10.0 % of the udder surface was soiled) as clean udders and scores three and four (> 10.0 % of the udder surface was soiled) as dirty udders to categorize them into two factors (clean vs. dirty). A herd was classified as a clean herd if > 80.0 % of the examined udders were assigned to score one or two (Table 1). A herd was classified as a dirty herd if ≤ 80.0 % of the examined udders were assigned scores of one or two (Table 1). The threshold was based on empirical values in a study by Schreiner and Ruegg from the year 2003 [39]. Other examined predictors are listed in Table 1.

Laboratory procedures: Conventional cytomicrobiological diagnostic examinations were performed at the laboratory of Hanover University of Applied Sciences and Arts, Hannover, Germany in accordance with the guidelines of the German Veterinary Association [36], which are comparable to the National Mastitis Council recommendations [40]. With a sterile calibrated loop, 10 µL of each well-mixed milk sample was plated on a quadrant of an aesculin blood agar plate (Thermo Fisher Scientific, Langenselbold, Germany). Plates were incubated for at least 48 h at 37 °C under aerobic conditions. Isolates were Gram stained to assist in organism identification. Furthermore, the morphology of colonies, aesculin hydrolysis, catalase reactivity (3 percent H_2O_2; Merck, Darmstadt, Germany), and hemolysis patterns were used for identification. Gram-positive and catalase-positive cocci were identified as staphylococci. For the differentiation of *S. aureus*, a clumping factor test was performed (Staph Plus Kit, DiaMondiaL, Vienna, Austria). Other staphylococci were referred to as non-aureus staphylococci (NaS). Gram-positive and catalase-negative cocci were identified as streptococci. For the differentiation of aesculin hydrolyzing cocci, modified Rambach agar was used [41]. Aesculin hydrolyzing and ß-d-Galactosidase-positive cocci were identified as *Str. uberis*. Aesculin hydrolyzing, ß-d-galactosidase-negative cocci were identified as enterococci. The ß-hemolytic streptococci were characterized by Lancefield serotyping (DiaMondiaL Streptococcal Extraction Kit, Sekisui Virotech GmbH, Rüsselheim, Germany). Streptococci from group C were referred to as *Str. dysgalactiae*, from group B as *Str. agalactiae*. Gram-positive, ß-hemolytic, catalase-negative irregular rods with V- or Y-shaped configurations were identified as *Trueperella* (*T.*) *pyogenes*. Gram-positive, catalase-positive, asporogenic colonies on aesculin blood agar were identified as coryneform bacteria. *Bacillus* species form colonies on aesculin blood agar which are catalase-positive and appear as Gram-positive rods forming endospores. Gram-negative and cytochrome oxidase negative (Bactident oxidase, Merck KGaA, Darmstadt, Germany) rods were further differentiated using Chromocult Coliform Agar (Merck KGaA). After incubation at 37 °C for 24 h, *E. coli* forms blue colonies and other coliforms form pink-red colonies. Gram-negative rods showing no mobility during the performance of the oxidative fermentative test were identified as *Klebsiella* species. Gram-negative, catalase-positive, and cytochrome oxidase-positive rod-shaped bacteria showing oxidative glucose degradation were identified as *Pseudomonas* species. Yeasts, molds, and *Prototheca* species were differentiated microscopically. Environmentally associated, mastitis-causing microorganisms (*Str. uberis, E. coli*, NaS, *Klebsiella* species, coliform bacteria, yeasts, *Pseudomonas* species, and *Prototheca* species) were recorded as a microbiologically positive result if ≥ 5 cfu/0.01 mL were cultured. Based on the recommendations of the National Mastitis Council [40], samples with two identified pathogens are covered by the definition of a mixed infection, whereas samples with more than two pathogens are described as contaminated, except if a colony of an *S. aureus*, *Str. agalactiae*, *Str. dysgalactiae*, or *T. pyogenes* was found [3].

Data from the Dairy Herd Improvement Tests: Data from the last DHI test prior to the onset of mastitis were evaluated on a herd-specific basis. General herd information such as the number of cows milking, total number of cows, average milking days per cow, the proportion of primiparous cows in the herd, average herd yield per cow per day, and average rolling 305-day yield per cow was recorded (Table 1). The herd milk composition, in terms of fat, protein, and urea content, was considered (Table 1). Enrolled data also included SCC in the last DHI test before a case of clinical mastitis as SCC per mL per cow, as SCC per mL from the diseased cow, and as average annual SCC per mL per cow from the herd (Table 1). The udder health report from the last DHI test prior to the onset of mastitis was utilized, including parameters such as the proportion of udder healthy dairy cows, the new infection risk (NIR) in lactation, the NIR in the dry period, and the heifer mastitis rate (Table 1). The udder health report was defined according to the Guidelines of the German Association for Performance and Quality Testing [42]. The proportion of udder healthy animals was calculated by dividing the number of dairy cows ≤ 100 kcells per mL in the DHI test by the number of all dairy cows in the DHI test [42]. The new infection risk in lactation was calculated by dividing the number of dairy cows that had ≤ 100 kcells per mL in the last DHI test and had > 100 kcells per mL in the following DHI test by the number of udder healthy animals in the last DHI test [42]. The NIR in the dry period was calculated by dividing the number of dairy cows that had ≤ 100 kcells per mL in the last DHI test before the dry period and had > 100 kcells per mL in the first DHI test after the dry period by the number of udder healthy animals (≤ 100 kcells) in the last DHI test before the dry period [42]. In a few dairy herds without NIR in the dry period in the last DHI test prior to mastitis, DHI test data could not be obtained until the start of the study, so we did not have all the necessary data retrospectively at the start of the study to capture NIR in the dry period correctly and completely. The heifer mastitis rate (HMR) was calculated by dividing the number of heifers that had > 100 kcells per mL in the first DHI test between day 5 and 30 after parturition by the number of all heifers in the DHI test [42].

Definition of the Outcome Variables: The outcome variables were the severity of CM and the incidence of severe mastitis. The definition of mastitis severity levels according to the International Dairy Federation was adjusted for statistical analysis into a binomial one, where MS 1 and MS 2 were defined as non-severe mastitis and MS 3 still represented severe mastitis. The incidence of severe mastitis per 100 cow years at risk was based on the number of lactating cows in the DHI test before mastitis and the study period [43]. All tested independent variables from the DHI tests related to the dependent variable of the incidence of severe mastitis were averaged from all DHI tests during the sample period. All predictors examined, including their definition and categorization, are listed in Table 1. Table 1 also lists the references that were the basis for the selection of these predictors and also define the cutoff values for many predictors.

Statistical analysis: For analyzing the dataset, the program SPSS 28.0,

IBM, Inc., Chicago, IL, USA, was used. Udder quarter with a clinical mastitis case was the statistical unit in the first model with the target variable of the severity of CM in the diseased dairy cow. Associations between the severity score of occurring CM and risk factors (independent variables) (Table 1) were examined with generalized linear mixed models with logit link and binomial response (severe/non-severe (logistic regression)) after pre-screening for variable selection in univariable analysis. In a second model, the incidence of severe mastitis was used as the outcome variable and herd-based factors were tested as explanatory variables. The relation between dependent and independent variables was tested first by appropriate univariable tests. Multicollinearity was checked with Spearman/Kendall's tau, which indicated a correlation of $r > 0.70$ with one another. For this reason, no variables were excluded. Then, independent variables associated with the dependent variable at $p < 0.10$ in the univariable test were submitted to generalized linear mixed models. Using logistic regression procedures, the association between severity and risk factors at herd level (independent variables) was examined. In the first model herd, cow within the herd, and quarter within a cow were considered random effects. A backward stepwise procedure was used to select the final multivariable regression model. Potential risk factors were excluded if $p > 0.05$. Meaningful biological interactions between the fixed effects were also used in the final model if significant ($p < 0.05$) and if they did not increase the Akaike information criterion (AIC). Non-significant effects or interactions that increased the AIC were not included in the final model. Model fit was evaluated by checking the normality of the residuals. Scaled identity was chosen as the covariance structure because it was assumed that there were no correlations between the elements. Odds ratios (OR) were calculated to describe the direction of the relationship between dependent and independent variables. OR were determined with 95 % confidence intervals (CI 95 %) and statistical significance was set at $p \leq 0.05$.

Results

Descriptive results: A total of 325 CM cases were enrolled in this cross-sectional study on 58 dairy farms in Northwestern Germany, located in NRW and Hesse, in the period between June 2020 and September 2020. A large proportion of all CM cases were caused by environment-associated microorganisms. The distribution of bacteriological findings is presented in Table 2, showing that the most frequently isolated pathogen group in all CM cases was coliform pathogens (32.6 %), followed by *Str. uberis* (17.2 %). Mastitis cases without pathogen growth were found to be the third most common among all CM cases (16.3 %).

Variables related to the severity of CM in the diseased dairy cow: In the consideration of the distribution of the severity of CM, 34.2 % (111 cases) of all CM cases had a mild course, 38.2 % (124 cases) had a moderate course, so that 72.4 % (235 cases) of all CM took a non-severe course and 27.6 % (90 cases) of all CM took a severe course (Table 2). The most common finding in severe cases of mastitis were coliform pathogens (52.2 %), followed by *Str. uberis* (15.6 %). The third finding in cases of severe mastitis was no growth and mixed infections (7.8 %). Moderate mastitis cases were also mostly caused by coliform pathogens (31.5 %), followed by *Str. uberis*. Mastitis without pathogen growth was the third finding among cases of moderate mastitis. In the mild cases, mastitis without pathogen growth was the most common finding (25.3 % of the cases), followed by NaS and *Corynebacterium* species, and coliform pathogens. Non-severe mastitis cases were mostly caused by coliform pathogens (25.1 %). The second and third most common findings in cases of non-severe mastitis were mastitis without pathogen growth and mastitis caused by *Str. uberis* (Table 2).

The distribution of the independent variable categories in severe CM cases in diseased dairy cows is listed in Table 3.

Variables related to the incidence of severe mastitis in the dairy herd: The incidences of severe mastitis in this study had a statistic range of 23.1 severe cases per 100 cow years at risk with a minimum of 0.0 severe cases per 100 cow years at risk and a maximum of 23.1 severe cases per 100 cow years at risk. The mean statistic for incidences of severe mastitis was 4.1 severe cases per 100 cow years at risk.

The mean incidence of severe mastitis cases was 6.0 severe cases per 100 cow years at risk among all dairy herds with a lower herd milk protein content < 3.4 % based on the average of all DHI tests during the sample period and 2.4 severe cases per 100 cow years at risk among all dairy herds with a herd milk protein content ≥ 3.4 %. The distribution

Table 2: Microbiological results from milk samples from udder quarters (n = 325) with clinical mastitis and their distribution by non-severe and severe clinical mastitis

	Mastitis severity score					
	Non-severe (MS1 + MS2)		Severe (MS3)		∑	
Microbiological findings	n	Proportion of the respective MS (%)	n	Proportion of the respective MS (%)	Total[1] (n)	Proportion of all cases (%)
Streptococcus (*Str.*) *uberis*	42	17.9	14	15.6	56	17.2
Coliform pathogens[2]	59	25.1	47	52.2	106	32.6
No growth	46	19.6	7	7.8	53	16.3
Non-aureus staphylococci (NaS) & *Corynebacterium* ssp.	34	14.5	3	3.3	37	11.4
Mixed infections	26	11.1	7	7.8	33	10.2
Staphylococcus (*S.*) *aureus*	12	5.1	4	4.4	16	4.9
Other[3]	6	2.5	5	5.6	11	3.4
Str. dysgalactiae	10	4.2	3	3.3	13	4.0
Total	235[4]	100	90[4]	100	325[5]	100

[1]number of cases per pathogen group with MS, [2]coliform pathogens: *Escherichia coli*, *Klebsiella* ssp., *Enterobacter*, [3]*Prototheca* spp., *Bacillus* spp., *Enterococcus* spp., yeast, *Pseudomonas* spp., *Streptococcus agalactiae*, other streptococci, *Trueperella pyogenes*, *Serratia* ssp., [4]number of non-severe and severe cases, [5]number of cases with recorded MS.

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Table 3, part 1: Distribution of the independent variable categories in severe clinical mastitis cases (n=90) in the diseased dairy cows in dairy herds (n = 58) in Northwestern Germany

		Severe CM cases (MS3) in the diseased dairy cow		Total[1]
Independent Variable		**n**	**%**	**n**
Udder cleanliness	Clean dairy herds	58	40.3	144
	Dirty dairy herds	32	17.7	181
Number of milkings	2 milkings / cow / day	38	21.5	177
	> 2 milkings / cow / day	52	35.1	148
NIR[2] dry period[3]	NIR ≤ 28.0 %	66	29.5	224
	NIR > 28.0 %	13	17.8	73
	No value	11	39.3	28
Pasturing	Pasturing	22	25.0	88
	No pasturing	68	28.7	237
Feed hygiene[4]	Feed hygiene	55	31.8	173
	No feed hygiene	35	23.0	152
Air conditioning	No air conditioning	55	30.1	183
	Air conditioning	35	24.6	142
Stocking density[4]	No overcrowding	65	33.2	196
	Overcrowding	25	19.4	129
Average herd milk yield / cow / day[3]	< 33 kg[5] / cow / day	27	18.9	143
	≥ 33 kg / cow / day	63	34.6	182
Average herd milk fat content / cow[3]	< 4.0 %	70	30.4	230
	≥ 4.0 %	20	21.1	95
Average herd milk protein content / cow[3]	< 3.4 %	57	38.8	147
	≥ 3.4 %	33	18.5	178
Average herd milk urea content / cow[3]	< 170 ppm[6]	25	33.3	75
	≥ 170 ppm	65	26.0	250
Average herd SCC[7] per mL[8] / cow[3]	< 200,000 SCC per mL / cow	37	29.8	124
	≥ 200,000 SCC per mL / cow	53	26.4	201
Proportion of udder healthy cows[3]	< 50.0 %	13	17.8	73
	≥ 50.0 %	77	30.6	252
NIR lactation[3]	NIR ≤ 21.0 %	55	29.3	188
	NIR > 21.0 %	35	25.5	137

of the independent variable categories in dairy herds (n = 23) with an incidence of severe mastitis above the mean statistical value of 4.1 severe cases per 100 cow years at risk is listed in Table 4.

Results of Mixed Regression Models:

Severity of CM in the diseased dairy cow: The relationship between the severity of CM in the diseased dairy cow and herd-related risk factors was analyzed using generalized linear mixed models with binomial response (severe and non-severe (logistic regression)), following a preliminary screening process to select variables in univariable analysis. The udder cleanliness of dairy herds was significantly associated with the severity of CM in the diseased dairy cow ($p < 0.001$) (Table 5). Mastitis cases in clean dairy herds had higher odds (OR 3.01, CI 1.63 – 5.55) of having severe mastitis than cases in dirty herds. The number of milkings per cow and day was significantly associated with the severity of CM in the diseased dairy cow ($p = 0.001$) (Table 5). Mastitis cases in dairy herds with > two milkings per cow and day had higher odds (OR 2.66, CI 1.51 – 4.68) of having severe mastitis than cases in herds with two milkings per cow and day. The NIR in the dry period in the last DHI test before the mastitis was associated with the severity of CM in the diseased dairy cow ($p = 0.009$) (Table 5). Mastitis cases in dairy herds with an NIR in the dry period ≤ 28.0 % in the last DHI test before the CM had higher odds (OR 2.61, CI 1.13 – 6.07) of having severe mastitis than cases in herds with NIR in the dry period > 28.0 %. Mastitis cases in dairy cows in herds where no NIR in the dry period in the last DHI test prior to CM were obtained also had higher odds (OR 6.57, CI 1.96 – 22.04) of developing a severe mastitis course than cases in dairy cows in herds with an NIR in the dry period > 28.0 %. The causative pathogen group of mastitis was positively associated with the severity of CM ($p = 0.027$) (Table 5) in the diseased dairy cow.

Incidence of severe mastitis in the dairy herd: The relationship between the incidence of severe mastitis (dependent variable) and herd-related risk factors (independent variables) was analyzed using a generalized linear mixed model. The herd milk protein content based on the average of all DHI tests during the sample period was significantly associated with the incidence of severe mastitis ($p = 0.005$) (Table 6). Dairy herds with a herd milk protein content < 3.4 % based on the average of all DHI tests during the sample period had higher odds (OR 38.15, CI 2.99 – 486.63) of having a higher incidence of severe mastitis

Table 3, part 2: Distribution of the independent variable categories in severe clinical mastitis cases (n=90) in the diseased dairy cows in dairy herds (n = 58) in Northwestern Germany

		Severe CM cases (MS3) in the diseased dairy cow		Total[1]
Independent Variable		**n**	**%**	**n**
Heifer mastitis rate[3]	≤ 40.0 %	82	30.4	270
	> 40.0 %	8	14.5	55
Average milking days / cow[3]	< 190	34	30.9	110
	≥ 190	56	26.0	215
Total starch content in the lactating ration[4]	≤ 20.0 %	34	21.9	155
	> 20.0 %	47	44.8	105
	No value	9	13.8	65
Feed changes[4]	No feed changes	65	28.6	227
	One or more feed changes	25	25.5	98
Total number of cows[3]	< 140	39	22.9	170
	≥ 140	51	32.9	155
Number of milking cows[3]	< 120	41	24.0	171
	≥ 120	49	31.8	154
Proportion of heifers in the herd[3]	< 33.0 %	59	29.4	201
	≥ 33.0 %	31	25.0	124
Average rolling 305-day milk yield / cow[9]	< 9543 kg / cow	19	19.8	96
	≥ 9543 kg / cow	71	31.0	229
Average annual herd SCC per mL / cow[9]	< 200,000 SCC per mL / cow	45	32.4	139
	≥ 200,000 SCC per mL / cow	45	24.2	186
SCC per mL of the diseased cow[3]	< 100,000 SCC per mL / cow	41	38.7	106
	≥ 100,000 SCC per mL / cow	49	22.4	219
Total		90[1]		325[10]

[1]number of severe CM cases in the diseased dairy cow, [2]new infection risk, [3]in the last dairy herd improvement test before the case, [4]in the last 14 days before the case, [5]kilogram, [6]part per million, [7]somatic cell count, [8]milliliter, [9]in the last DHI test control year, [10]number of cases with recorded MS.

than dairy herds with a herd milk protein content ≥ 3.4 %.

Discussion

The aim of this study was, on the one hand, to elaborate risk factors at herd-level in association with severe CM in the diseased dairy cow. On the other hand, another objective was to investigate herd-related factors associated with the incidence of severe mastitis in the dairy herd.

Severity of CM in the diseased dairy cow: Udder cleanliness was associated with the severity of CM in the diseased dairy cow. Mastitis cases in clean dairy herds were associated with more severe mastitis in relation to the reference category of mastitis cases in dirty herds. The fact that mastitis cases in clean dairy herds were positively associated with severe CM in the diseased dairy cow, whereas clean herds were not associated with a higher incidence of severe mastitis provides possibilities for interpretation. Therefore, the risk of developing severe mastitis is lower for dirty dairy cows only because there are more non-severe mastitis cases occurring in these animals. There may be a very different pathogen distribution in mastitis cases in dirty herds than in cases in clean herds. Preexisting pathogens in the udders of dirty dairy cows, such as minor pathogens and cow-associated pathogens, result in higher SCC > 100k, which are associated with subclinical, chronic, or mild mastitis [23, 56]. Dirty herds were associated with higher BMSCC [39]. High BMSCC may be indicative of high floods of PMN in the udder in response to pathogens. Overcrowding can result in excessive contamination, which often increases the exposure to environmentally associated pathogens such as *E. coli* and the incidence rate of mastitis caused by these pathogens [12, 14, 23, 24]. Dirty dairy cows have more mild and moderate mastitis due to environmentally associated pathogens and therefore relatively less severe mastitis. Overcrowding can also lead to increased stress levels, which, in turn, have an immunosuppressive effect. In clean herds there are more healthy udder quarters and dairy cows, i.e., the udder quarters and dairy cows are healthy for a longer time. Healthy udders with a low SCC < 100k may have insufficient protection against pathogens [18]. Low BMSCC, which may be an indirect indicator of many udder healthy cows in a dairy herd, may be associated with clean herds and with a significantly higher incidence of moderate and severe mastitis [13, 39]. In udder healthy cows with low SCC, the immune system may not be activated and thus may not be prepared for invading pathogens, so pathogen elimination can only occur with a time delay. This could allow the invading pathogens to multiply more quickly, as evidenced by higher pathogen shedding in severe cases of mastitis [2]. A high number of pathogens in the udder quarter may then be followed by an increased immune response, which may be clinically visible in more severe local and systemic signs of inflammation. Related to the group of pathogens causing mastitis, mastitis can potentially be more severe when many major pathogenic microorganisms encounter healthy udder quarters.

The number of milkings per dairy cow and day was associated with the severity of CM in the diseased dairy cow. Mastitis cases in dairy herds with > two milkings per dairy cow and day were positively associated

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with severe mastitis in relation to the reference category of cases in herds with two milkings per dairy cow and day. The fact that mastitis cases in dairy herds with > two milkings per dairy cow and day were positively associated with severe CM in the diseased dairy cow, whereas herds with > two milkings per dairy cow and day were not associated with a higher incidence of severe mastitis provides possibilities for interpretation. Therefore, the risk of developing severe mastitis is lower for herds with two milkings per cow and day only because there are more non-severe mastitis cases in herds with two milkings per cow and day. A large proportion of dairy farms with > two milkings per cow and day milked with automatic milking systems (AMS) and were thus classified as robot herds. Bausewein *et al.* (2022) reported in their study that less mild and moderate cases of mastitis were detected on dairy farms with AMS [44]. Mild mastitis may be noticed or investigated later and sampled less frequently in robot herds. The proportion of cases of severe mastitis of the total number of cases of mastitis on dairy farms with > two milkings per cow and day was higher compared to farms with two milkings per cow and day. There is a presumption that severe mastitis is detected faster in robot herds due to the technical support. Dairy cows with a higher milking frequency could be at higher risk of infection for mastitis from environmentally associated pathogens compared to dairy cows with a lower milking frequency because the teat canal is open for a longer time during the day and the teat tissue has less time to recover in the shortened intermediate milking period [45]. Furthermore, increased flushing of pathogens due to more frequent milking could be a factor [45]. On dairy farms with AMS, the intermilking period can be highly variable, so in addition to these shortened intermilking periods, extended intermilking periods can occur, which allow invading pathogens a longer time to multiply [45]. In contrast, another explanation is that dairy herds with two milkings per cow and day have a lower risk of developing mastitis due to cow-associated pathogens compared with herds with > two milkings per cow and day, but conversely have a higher risk of developing mild and moderate mastitis due to environmentally associated pathogens and therefore relatively less severe mastitis. In this study, conventionally milking dairy farms with more than two milkings per animal per day were two

Table 4, part 1: Distribution of the independent variable categories in dairy herds (n = 23) with an incidence of severe mastitis above the mean statistical value of 4.1 severe cases per 100 cow years at risk in dairy herds (n = 58) in Northwestern Germany

		Dairy herds with an incidence of severe mastitis above the mean statistical value of 4.1 severe cases per 100 cow years at risk		Total[1]
Independent Variable		**n**	**%**	**n**
Udder cleanliness	Clean dairy herds	11	52.4	21
	Dirty dairy herds	12	32.4	37
Number of milkings	2 milkings / cow / day	11	29.7	37
	> 2 milkings / cow / day	12	57.1	21
NIR[2] dry period[3]	NIR ≤ 28.0 %	13	34.2	38
	NIR > 28.0 %	4	33.3	12
	No value	6	75.0	8
Pasturing	Pasturing	9	52.9	17
	No pasturing	14	34.1	41
Feed hygiene	Feed hygiene	12	44.4	27
	No feed hygiene	11	35.5	31
Air conditioning	No air conditioning	18	47.4	38
	Air conditioning	5	25.0	20
Stocking density	No overcrowding	17	50.0	34
	Overcrowding	6	25.0	24
Average herd milk yield / cow / day[3]	< 33 kg[4] / cow / day	14	42.4	33
	≥ 33 kg / cow / day	9	36.0	25
Average herd milk fat content / cow[3]	< 4.0 %	16	44.4	36
	≥ 4.0 %	7	32.8	22
Average herd milk protein content / cow[3]	< 3.4 %	15	53.6	28
	≥ 3.4 %	8	26.7	30
Average herd milk urea content / cow[3]	< 170 ppm[5]	6	46.2	13
	≥ 170 ppm	17	37.8	45
Average herd SCC6 per mL[7] / cow[3]	< 200,000 SCC per mL / cow	7	36.8	19
	≥ 200,000 SCC per mL / cow	16	41.0	39
Proportion of udder healthy cows[3]	< 50.0 %	4	30.8	13
	≥ 50.0 %	19	42.2	45
NIR lactation[3]	NIR ≤ 21.0 %	15	45.5	33
	NIR > 21.0 %	8	32.0	25

Table 4, part 2: Distribution of the independent variable categories in dairy herds (n = 23) with an incidence of severe mastitis above the mean statistical value of 4.1 severe cases per 100 cow years at risk in dairy herds (n = 58) in Northwestern Germany

		Dairy herds with an incidence of severe mastitis above the mean statistical value of 4.1 severe cases per 100 cow years at risk		Total[1]
Independent Variable		**n**	**%**	**n**
Heifer mastitis rate[3]	≤ 40.0 %	19	42.2	45
	> 40.0 %	4	30.8	13
Average milking days / cow[3]	< 190	7	36.8	19
	≥ 190	16	41.0	39
Total starch content in the lactating ration	≤ 20.0 %	9	42.9	21
	> 20.0 %	7	43.8	16
	No value	7	33.3	21
Total number of cows[3]	< 140	14	37.8	37
	≥ 140	9	42.9	21
Number of milking cows[3]	< 120	14	38.9	36
	≥ 120	9	40.9	22
Proportion of heifers in the herd[3]	< 33.0 %	15	41.7	36
	≥ 33.0 %	8	36.4	22
Average rolling 305-day milk yield / cow[3]	< 9543 kg / cow	11	50.0	22
	≥ 9543 kg / cow	12	33.3	36
Average annual herd SCC per mL / cow[8]	< 200,000 SCC per mL / cow	12	50.0	24
	≥ 200,000 SCC per mL / cow	11	32.4	34
Total	23[1]			58[9]

[1]number of dairy herds with an incidence of severe mastitis above the mean statistical value of 4.1 severe cases per 100 cow years at risk, [2]new infection risk, [3]based on the average of all dairy herd improvement tests during the sample period, [4]kilogram, [5]part per million, [6]somatic cell count, [7]milliliter, [8]in the last dairy herd improvement test control year based on the average of all dairy herd improvement tests during the sample period, [9]number of dairy herds with recorded incidence of severe mastitis.

larger herds with approximately 260 and 390 dairy cows. The milkers on these dairy farms were predominantly hired personnel with limited specialized apprenticeships and with a high turnover. Here, the quality and quantity of mastitis diagnostics could possibly be an influencing factor. High turnover of milking personnel with changing milking routines can also be a stressor for dairy cows.

The NIR in the dry period in the last DHI test before the mastitis was associated with the severity of CM in the diseased dairy cow. Mastitis cases in dairy herds with NIR in the dry period ≤ 28.0 % in the last DHI test prior to mastitis were positively associated with severe mastitis in relation to the reference category of cases in herds with NIR in the dry period > 28.0 %. Mastitis cases in dairy herds where no NIR in the dry period in the last DHI test prior to CM were obtained were also positively associated with severe mastitis in relation to the reference category of cases in herds with NIR in the dry period > 28.0 %. In a few dairy herds without NIR in the dry period in the last DHI test prior to mastitis, DHI test data could not be obtained until the start of the study, so we did not have all the necessary data retrospectively at the start of the study to capture NIR in the dry period correctly and completely. The average dairy farm of the North-Rhine Westphalia State Control Association had an NIR in the dry period of 28.0 % in an annual report from 2020 based on DHI tests [34]. Mastitis was more likely to be severe when dairy herds had NIR in the dry period ≤ 28.0 %. Mastitis cases in dairy herds with NIR in the dry period ≤ 28.0 % in the last DHI test prior to mastitis were positively associated with severe CM in the diseased dairy cow, whereas herds with NIR in the dry period ≤ 28.0 % in the last DHI test prior to mastitis were not associated with a higher incidence of severe mastitis. Thus, the risk of developing severe mastitis was lower for herds with NIR in the dry period > 28.0 % only because there were more non-severe mastitis cases in herds with NIR in the dry period > 28.0 %. It is possible that there is a difference in pathogen distribution between dairy herds with NIR in the dry period ≤ 28.0 % and > 28.0 %, such that in herds with NIR in the dry period > 28.0 %, other pathogens are already present in the udder quarters. The dairy cow's immune system responds to these pathogens present with increased PMN in the udder quarter and ultimately increased SCC in the milk. Thus, more subclinical, chronic, or mild and moderate cases of mastitis due to environmentally associated pathogens may be present in dairy herds with NIR in the dry period > 28.0 % and therefore relatively less severe mastitis. Only healthy udder quarters can reinfect in the first place, resulting in more udder healthy animals on dairy farms with NIR in the dry period ≤ 28.0 %. Again, there could be an association with severe mastitis when major pathogenic microorganisms encounter healthy udder quarters. During the dry period, the immune system is suppressed by metabolic and hormonal changes [14, 15]. The association between dairy farms where no NIR in the dry period in the last DHI test prior to CM were obtained and severe mastitis could be a random effect of these few affected dairy farms, or could come about if these dairy farms did not notice and sample every mild mastitis case.

The group of pathogens causing mastitis was associated with the severity of the CM in the diseased dairy cow. The number of samples in this study was too small for specific and reliable statements regarding individual pathogen groups and their association with the severity of CM. However, coliform pathogens showed higher odds of causing severe mastitis compared to other pathogens in relation to the reference category of *Str. dysgalactiae*. More than half (52.2 %) of all severe

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Table 5: Final generalized linear mixed model with binominal response for the severity of clinical mastitis (n = 325) in the diseased dairy cow in herds (n = 58) in Northwestern Germany

Effect		ß[1]	SE[2]	t value	OR[3]	95 % CI[2] (OR)	*p*-value
Udder cleanliness							< 0.001
	Clean dairy herds	1.10	0.32	3.54	3.01	1.63 – 5.55	< 0.001
	Dirty dairy herds						Reference
Number of milkings							0.001
	2 milkings / cow / day						Reference
	> 2 milkings / cow / day	0.98	0.29	3.42	2.66	1.51 – 4.68	0.001
NIR[5] dry period[6]							0.009
	NIR ≤ 28 %	0.96	0.43	2.25	2.61	1.13 – 6.07	0.025
	NIR > 28 %						Reference
	No value	1.88	0.62	3.06	6.57	1.96 – 22.04	0.002
Pathogen group							0.027
	Non-aureus staphylococci (NaS) & *Corynebacterium ssp.*	-1.28	0.96	-1.34	0.28	0.04 – 1.83	0.182
	Staphylococcus (*S.*) *aureus*	0.05	0.96	0.05	1.05	0.16 – 6.98	0.958
	Streptococcus (*Str.*) *uberis*	-0.17	0.80	-0.21	0.84	0.17 – 4.10	0.831
	Coliform pathogens[7]	0.53	0.75	0.70	1.70	0.38 – 7.48	0.484
	Mixed infections	-0.51	0.87	-0.59	0.60	0.11 – 3.30	0.554
	No growth	-0.93	0.84	-1.11	0.39	0.08 – 2.05	0.267
	Other[8]	0.74	1.03	0.72	2.10	0.28 – 15.79	0.471
	Str. dysgalactiae						Reference

[1]regression coefficient, [2]standard error of the mean, [3]odds ratio, [4]95 % confidence interval for OR, [5]new infection risk, [6]in the last dairy herd improvement test before the case, [7]coliform pathogens: *Escherichia coli*, *Klebsiella* ssp., *Enterobacter*, [8]*Prototheca* spp., *Bacillus* spp., *Enterococcus* spp., yeast, *Pseudomonas* spp., *Streptococcus agalactiae*, other streptococci, *Trueperella pyogenes*, *Serratia* ssp..

mastitis cases in this study were caused by coliform pathogens. Other studies have also shown associations between coliform pathogens such as *E. coli* and more severe courses of mastitis [11, 13]. In particular, high numbers of coliform pathogens such as *E. coli* were associated with more severe CM [2, 14]. A high coliform count may indicate either an extremely high pathogen load of coliform pathogens or a sign of decreased elimination of pathogens by PMN due to immunosuppression [2]. The dairy cow's risk of developing mastitis in a dairy herd is composed of the pathogen load and the immune system's ability to eliminate these pathogens [17]. Endotoxins, which are produced when the outer cell membrane of Gram-negative pathogens disintegrates, can cause strong immune reactions [57]. These endotoxins trigger the cytokine tumor necrosis factor alpha (TNF-α) [57]. Severe manifestations of mastitis may be related to extremely high loads of endotoxins or strong host immune responses [58]. The pathogen *Str. uberis* caused 15.6 % of severe mastitis cases, making it the second most common pathogen. Environmentally associated pathogens were causative for a large proportion of severe CM.

Table 6: Final generalized linear mixed models with the incidence of severe mastitis (dependent variable) in herds (n = 58) in Northwestern Germany

					95 % CI[4] (OR)		
Independent variable		**ß[1]**	**SE[2]**	**OR[3]**	**Lower**	**Upper**	***p*-value**
Average herd milk protein content / cow[5]							
	< 3.4 %	3.64	1.30	38.15	2.99	486.63	0.005
	≥ 3.4 %						Reference

[1]regression coefficient, [2]standard error of the mean, [3]odds ratio, [4]95 % confidence interval for OR, [5]based on the average of all dairy herd improvement tests during the sample period.

Incidence of severe mastitis in the dairy herd: In a follow-up step, we examined the independent variables for a possible association with the outcome variable of the incidence of severe mastitis to find out why dairy herds differ in absolute incidence of severe mastitis cases. The herd milk protein content based on the average of all DHI tests during the sample period was significantly associated with the incidence of severe mastitis in the dairy herd. Dairy herds with a lower herd milk protein content < 3.4 % based on the average of all DHI tests during the sample period were associated with a higher incidence of severe mastitis. Thus, in herds with a herd milk protein content < 3.4 % there occurred absolutely more severe mastitis cases. The mean incidence of severe mastitis cases was 6.0 severe cases per 100 cow years at risk among all dairy herds with a lower herd milk protein content < 3.4 % based on the average of all DHI tests during the sample period and 2.4 severe cases per 100 cow years at risk among all dairy herds with a herd milk protein content ≥ 3.4 %. Possible explanations could be that the herd protein content is an indicator of the energy supply of the dairy herd [59, 60]. A herd protein content of ≥ 3.4 should be aimed for. Dairy herds with protein contents < 3.4 % are energy deficient. As the immune system, especially when activated, requires a lot of energy to function efficiently, dairy herds may be immunosuppressed when energy is deficient [19] and the number and function of PMN may be reduced. In phases of immunosuppression, the invaded pathogens can multiply more quickly, as evidenced by higher pathogen shedding in severe mastitis [2]. A high number of pathogens in the udder quarter

is then followed by an increased immune response, which is clinically visible in more severe local and systemic signs of inflammation. A good energy supply is essential for a well-functioning immune system and thus for rapid elimination of pathogens in the udder quarter. With regard to the risk factor of early lactation for severe mastitis [2, 14, 15], no association was shown at herd-level between average milking days as an indirect indicator of the proportion of dairy cows in early lactation in the total herd and the severity of CM. The proportion of first lactation dairy cows in the total herd is a possible indirect indicator of dairy cow parity, which was associated with mastitis severity in some studies [14, 15]. In the present study, no association was shown between the proportion of primiparous dairy cows in the last DHI test before mastitis and the severity of CM.

The severity of CM in this study was divided into 34.2 % mild mastitis cases, 38.2 % moderate mastitis cases, so that 72.4 % (235 cases) of all CM took a non-severe course, and 27.6 % (90 cases) of all CM a severe course. The proportion of severe mastitis to total mastitis in this study was higher than in other studies [2, 3, 11]. There are two explanations for the higher percentage of severe mastitis cases in the present study. First, the sample period, which was four warm summer months, could be a factor. In warm summer months, the absolute number of cases of severe mastitis could be higher due to a weakened immune system and a higher pathogen load. Second, not all dairy farms in this study may have noticed and sampled every case of mild mastitis, especially since higher numbers of severe mastitis require higher levels of care. Less mild and moderate mastitis cases are recorded in robot herds [44]. In the pathogen distribution among all mastitis cases in our study, the most frequently detected pathogen group was coliform pathogens, accounting for 32.6 %. The second most common pathogen in relation to all mastitis cases in the study was *Str. uberis* (17.2 %). Besides the environment-associated mode of transmission of *Str. uberis*, there is mounting evidence of a possible contagious transmission among cows [61]. The third finding was no pathogen growth (16.3 %) in mastitis cases. Compared with other studies, environmentally associated pathogens were also mainly responsible for CM, but *Str. uberis* was not the dominant pathogen [2, 3, 5]. One reason for the high incidence of coliform pathogens in the present study could be the sample period of the warm summer months. Warm temperatures and high humidity could increase coliform pathogen multiplication rates, leading to higher bacterial loads of coliform pathogens.

In this study, it was infeasible to examine all known risk factors in association with the severity of CM. Only one farm used a mastitis-associated vaccination, so it was not possible to comment on a possible association between mastitis-associated vaccination and mastitis severity. Testing associations between micronutrient supply and CM severity was not viable in this study design. The selected dairy farms were localized in Northwestern Germany, especially in the districts of Hochsauerlandkreis, Soest, Paderborn, Märkischer Kreis, and Waldeck-Frankenberg. The NRW State Control Association reported in an annual review an average farm size in South Westphalia of 87.5 cows per farm with an average rolling 305-day milk yield per cow of 9,543 kg and a herd average SCC of 214 kcells per mL based on DHI tests [39]. In northern Germany and the Lower Rhine region, the herd size of dairy farms tends to increase, but the performance parameters are similar on average across farms. When discussing strengths and weaknesses of this study, a longer sample period could be chosen for research on the influence of herd-specific risk factors on the severity of CM and more dairy herds could be chosen for research on herd-specific risk factors relating to the incidence of severe mastitis. Further research is needed to verify whether there are associations between feeding parameters and the severity of CM.

Conclusion

In this study, several herd-related factors associated with the severity of CM in the diseased dairy cow and associated with the incidence of severe mastitis in the dairy herd could be elaborated. Mastitis cases in clean dairy herds were associated with more severe mastitis. Mastitis cases in dairy herds with > two milkings per cow per day were positively associated with more severe mastitis cases. The NIR in the dry period in the last DHI test before the mastitis was associated with the severity of CM in the diseased dairy cow, so mastitis cases in dairy herds with NIR in the dry period ≤ 28.0 % showed positive associations with severe mastitis. The causative pathogen group was another risk factor in positive association with severe CM in the diseased dairy cow. The mean incidence for severe mastitis in this study was 4.1 severe cases per 100 cow years at risk. The herd milk protein content based on the average of all DHI tests during the sample period was significantly associated with the incidence of severe mastitis in the dairy herd, such that dairy herds with a lower herd milk protein content < 3.4 % were associated with a higher incidence of severe mastitis.

Disclosure of Conflicts of Interest

The authors declare no potential conflicts of interest.

Compliance with Ethical Standards

All applicable guidelines for the care and use of animals were followed. This study was conducted in compliance with ethical standards reviewed by the Animal Welfare Committee of the university (University of Veterinary Medicine Hannover, Foundation, Hannover, Germany; file reference: TVO-2020-V-63). The date when ethical approval was obtained was June 3rd, 2020. An application for a license for animal testing was not required by the local government due to the study design. The study complied with the International Guiding Principles for Biomedical Research Involving Animals (1985).

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