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REGULAR ARTICLES

Prevalence of bovine mastitis and main risk factors in Tunisia

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Abstract
Tunisian milk chain is in danger with a dramatical reduction of milk production over the last years. Improving the quality of milk seems mandatory to improve farmers' income, but for the moment, there is only scarce data on milk quality management in Tunisia. In this context, the aims of our study were first to describe the prevalence of bovine mastitis in 267 cows from 71 representative small dairy farms in the North (43.7%) and Center (56.3%) regions of Tunisia, using mastitis detection by California Mastitis Test (CMT) on milk quarter and clinical signs, and second, to assess possible risk factors for mastitis from animal, environment, and breeding management. In parallel, cow and bulk milk somatic cell count (SCC) were analyzed. Our results demonstrated that 60.3% of cows showed mastitis as determined by CMT and clinical examinations. Increased stage of lactation, parity, udder depth, and type of milking were significantly ($P < 0.05$) associated with increased odds of mastitis prevalence. The mean of individual cow SCC (ISCC) and bulk milk SCC (BMSCC) was very high (1083×10^3 cells/mL and 698×10^3 cells/mL, respectively), all ranks and stages of lactations combined. These high values confirm the infectious origin of mastitis that we found caused mainly by *Staphylococcus aureus* and coagulase-negative Staphylococci. In conclusion, control of the identified risks factors and improved biosecurity measures must be encouraged to restore udder health and milk quality and thus productivity and durability of Tunisian milk chain.

Keywords Udder health · Risk factors · Dairy cows · Milking · Tunisia

Introduction

Tunisian milk chain is in danger due to low mean milk productivity (3200 L/cow/lactation in 2017) which threatens the supply of the local market when the demand of milk and dairy products continues to increase (109.9 L/inhabitant) (GIVLAIT 2018). Economical balance is now again negative with increased imports (+ 16,300 Tons in 2017, Chebbi et al. 2019) which had been improved over the last 15 years through a strong public dairy policy. More than 83% of the Tunisian dairy farmers have very little farms and flocks (less than 5 dairy cows, Sakly et al. 2014). Due to low forage productivity and poor food autonomy, farmers use a high percentage of concentrates resulting in increasing production costs (Jaouad 2010; Hammami et al. 2017). That leads to the abandonment of production by an increasing number of producers. While low reproductive performances are well described in Tunisia (Ben-Salem et al. 2006; M'Hamdi et al. 2010) with important consequences on numeric productivity of flocks, mastitis remains the other most common problem of dairy cows able to explain this poor productivity and consequences. Mastitis causes a lot of economic losses on several levels, either by the decrease in milk production (Mtaallah et al. 2002; Hagnestam et al. 2007; Schneider et al. 2007), by the deterioration of the milk quality (Guérin-Faublée et al. 2003) and decrease in high value component concentrations as casein, lactose, and fat (Larsen et al. 2010; Vidanarachchi et al. 2015) or by the

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premature culling and needed replacement of the incurable cows (Aghamohammadi et al. 2018).

Thus, up-to-date and available information on mastitis prevalence and risk factors associated with the disease are important for udder health management of cow on the farm. In addition, identification of pathogens associated with mastitis is essential to confirm the contagious or environmental origin of mastitis (Quinn et al. 2002; Andrews et al. 2003), to better treat infected animals, and to reduce the infection incidence.

A vast range of microorganisms can cause mastitis in cattle, with the main pathogens historically responsible for the majority of cases are staphylococci (*Staphylococcus aureus* and Coagulase negative Staphylococci), *Streptococcus uberis*, and *Escherichia coli* (Oliveira et al. 2007; Fadlelmula et al. 2009; Pyörälä and Taponen 2009; Mekonnen et al. 2017; Vakkamäki et al. 2017). Studies on the etiology of mastitis in Tunisia are almost absent except a few studies on the antimicrobial resistance of mastitis pathogens (Saidani et al. 2018; Klibi et al. 2019).

Mastitis in dairy herds results from a complex interaction between host, environment, and agent. Several studies reported that mastitis were associated with individual cow risk factors including breed, parity, age at first calving, high milk production, milk leakage, udder edema, and reproductive disorders (Schukken et al. 1990; Oltenacu and Ekesbo, 1994; Peeler et al. 2000; Piepers et al. 2011) and with management-related herd factors, such as farm and milking hygiene, milking technique, housing, and feeding (Barkema et al. 1999). Evaluation of mastitis prevalence and characterization of risk factors were made in numerous studies in African countries (Bouzid et al. 2011; Saidi et al. 2013; Abebe et al. 2016; Mekonnen et al. 2017; Ndahetuye et al. 2019). Nevertheless, there are almost no studies about mastitis prevalence and risk factors in the dairy Tunisian production except works of M'sadak et al. (2013, 2015, 2016) on farms of Sahel region of Tunisia and work of Mtaallah et al. (2002) on large-scale farms.

Therefore, the objectives of this study were first to evaluate the prevalence of mastitis at cow level in smallholder dairy farms, more representative of dairy landscape in Tunisia. Second, to identify causative pathogens and to assess the mastitis risk factors due to animals (udder traits, animal cleanliness), environment (housing cleanliness), and breeding management (production system, milking practices).

Materials and methods

Study area and sample size

The study was carried out on 71 small dairy farms in the North (Bizerte, Beja, and Jendouba) and Center East (Mahdia) regions of Tunisia. These two areas are the main contributors to milk production in Tunisia. The farms selected are small representative farms with a low and medium number of dairy cows of different breeds. The total number of lactating cows in the studied farms was 267 with an average of 4 cows per farm and at DIM varying between 12 and 510 days, the most dominant breeds being Holstein, Brown Swiss, and crossed (Holstein × Brown Swiss). This study was carried out during the hot season from April to September with an average temperature of 27.7 °C and average temperature humidity index (THI) of 75.4. The choice of farms was made randomly. Farms were characterized by two types of milking, either mechanical ($n = 241$) or manual ($n = 26$), with a frequency of two milking per day.

The sample size was determined according to the cluster sampling formula (Bennett et al. 1991): $n = p(1-p)DZ^2/e^2b$, where n is the sample size (number of smallholder dairy farms); p = estimated prevalence of mastitis; e = desired absolute precision; b is number of lactating dairy cows to be sampled from each farm; Z = the alpha value of 95% CI = 1.96; D = design effect with D = 1 + (b-1) roh, where roh is a rate of homogeneity which was estimated at 0.02. The sample size was determined at 95% confidence level, 6% precision, D = 1.06, b = 4, and with an expected prevalence of 50%. A total of 71 small dairy farmers were thus selected to participate in the study.

Sampling technique

A multi-stage sampling procedure was adopted to select participating dairy farmers. Initially, we chose four governorates according to their different contribution to total milk production in Tunisia and their different concentrations in small-scale breeders. Three governorates in the north (the first dairy basin) and one in the center (second dairy basin) were selected. Secondly for the northern governorates, a purposive selection of the study areas was based on the fact that they are under control of the ODESYPANO breeder organization (Western North Sylvo-Pastoral Development Office) in each governorate for better availability of data and facility of access to breeders: We chose two areas in Beja and Bizerte and one in Jendouba with the greater number of controlled breeders. For the central eastern governorate (Mahdia), we randomly chose an area under the intervention of a mutual agricultural services company. In the third stage, a total of 31 (12.6%) small farmers were randomly sampled in the northern governorate from an established sampling frame of all controlled small dairy farmers (246) in the selected areas using a simple random sampling procedure. In the central-eastern governorate, a total of 40 (7.3%) small farmers were randomly sampled from all controlled small dairy farmers (547) in the selected area. Finally, a total of 71 smallholders were randomly sampled using simple

random sampling and all lactating dairy cows were included in the study (267 lactating cows, 133 in the north and 134 in the center).

Data collection

An epidemiological survey was carried out on the farms studied. It included detailed questions to obtain information on breeder, cow description (breed, parity, and physiological stage), zootechnical parameters, and breeding management. The survey was completed by interviewing the breeder and attending all steps of milking.

In addition, the milking machine was evaluated visually by the description of the general condition of the milking equipment such as the liner status, the tubes status, and the claw status. Cleanliness status of milking machine was estimated by the cleaning method used and by the presence of milk fat residues or milk clots in the claws, liner, short and long milk tubes, or in buckets. The survey data was collected into Microsoft Excel spreadsheets, until statistical analysis.

Mastitis detection and collection of milk samples

Clinical inspection

Before the collection of milk samples, the diagnosis of clinical mastitis was made by physical examination of the udder and teats (visualization and palpation) and by visual examination of foremilk in a bowl with black bottom. A quarter was considered having clinical mastitis if it was swollen and/or painful and/or had a visible injury or lesion and/or if the milk showed changes in the appearance (color) and/or the consistency (clots, flakes or blood). A cow with at least one positive quarter was classified as positive for clinical mastitis.

California Mastitis Test

CMT test was used for screening subclinical mastitis directly in the barn and to calculate subclinical mastitis prevalence. Procedures described by the National Mastitis Council (1999) were followed. After foremilking, approximately 2 mL of milk from each quarter was mixed with an equal volume of CMT reagent. The CMT results were scored (0 and 1 (negative), + 2, + 3, and + 4 (positive)) based on milk viscosity and the degree of gel formation (Berthelot et al. 1987). A cow with at least one positive quarter (score ≥ 2) was classified as positive for subclinical mastitis.

Somatic cell concentrations

Somatic cell concentrations were measured on the bulk milk (BMSCC) and on the individual cow milk (ISCC). Bulk raw milk samples from each farm were taken from a representative combination of bucket milk of the different cow milked per farms, while the individual cow milk samples were taken from a milk combination of the four udder quarters (a composite milk sample) obtained manually after forestripping. Samples from the evening milking were collected following National Mastitis Council guidelines (NMC 1999), in sterile plastic bottles (approximately 30 mL) containing bronopol as a preservative and kept around 2 °C to be transferred to local laboratories for the determination of somatic cell concentrations (SCC) in milk, using an automatic analyzer (Fossomatic 4000®). Somatic cell count was converted to somatic cell score (SCS) in order to provide normal distribution according to the following formula: $SCS = \log2(SCC/100{,}000) + 3$ (Ali and Shook 1980).

Bacterial isolation and identification

All positive milk samples, collected aseptically according to the National Mastitis Council (NMC 2017) from cows with subclinical mastitis (score CMT ≥ 2) or with clinical mastitis, were subjected to bacteriological examination. Microbiological analysis was performed in the diagnostic bacteriology laboratory at Veterinary Research Institute of Tunis (IRVT).

To identify bacteria in milk samples, 10 µL of milk were plated onto blood agar (tryptone soy agar with 5% sheep blood) and on BCP agar (BromoCresolPourpre). Subsequently, these plates were incubated for 24–48 h at 37 °C. Afterwards, an evaluation of colony morphology was made (hemolytic activity and form) and each type of colony was subjected to gram staining to define the gram (+) and gram (−) bacteria and thus choose the type of rapid identification gallery (API20E, API20NE, API20Staph, API20Strep, bioMérieux) to use. In addition, other tests have been carried out such us catalase, oxidase, coagulase, and mobility tests. A cow was considered infected if it was diagnosed as having mastitis and 1 or 2 pathogens were isolated from the milk sample.

Cow conditions and housing cleanliness

Cleanliness of animal was scored by referring to the grid of Faye and Barnouin (1985). The following anatomical zones were scored: ano-genital region, udder rear view, udder side view, and region of the leg-hock.

Housing cleanliness was indirectly evaluated by scoring the same anatomical areas and adding cleanliness of the entire thigh surface.

The scores assigned to each zone varied from 0 (no soils) to 2 (area completely soiled or covered with a thick crust).

Udder morphology

The udder morphology was evaluated by udder scoring using qualitative assessment of the udder morphology and by semi quantitative scoring (scale from 1 to 9) of some morphological traits according to the table used by the World Holstein Friesian Federation (2005). Measurements were taken before milking by the same person. Among the different zones described, the udder depth, udder front-rear balance, and height of the udder rear attachment were tested to determine their effects on the prevalence of mastitis. Udder depth is the distance between the udder floor and the hock joint. It was determined by measuring the udder floor as below or above the hock or at the hock level. The height of the udder rear attachment was measured as the distance from the bottom of the vulva to the base of the rear udder (Singh et al. 2014). The distance can be very low (< 20 cm), intermediate (20–25 cm), or very high (> 25 cm). Udder front-rear balance is the distance between two horizontal lines each passing by the base of the front and rear teats. If the rear quarters are very high compared to front quarters and vice versa, the udder is considered unbalanced, and if the base of the front and rear teats is placed on a horizontal line, the udder is balanced.

Statistical analysis

All data were exported to SPSS, version 20.0 for statistical analysis. The response variable selected for review in the statistical analysis was mastitis prevalence. It was calculated as the proportion of mastitis-positive cows (as defined by CMT test and clinical signs) in the total number of cows investigated. The association between the dependent variable, cow mastitis prevalence (0 = negative and 1 = positive), and each independent variable (risk factors) was investigated using univariable logistic regression analyses. The independent variables evaluated were region (North, Center East), production system (semi intensive (cows grazed on pasture and received concentrate feeds, hay, straw, and green fodder as complements), off-ground (animal feed is mainly purchased from outside; hay, straw, fodder with a larger percentage of concentrate in ration. It is often considered as intensive but here with low productivity)], breed (Holstein, Brown Suisse, cross breed, and others), lactation stage (early, ≤ 60 DIM; mid, > 60–210 DIM; and late lactation, > 210 DIM), parity (primiparous vs multiparous), cow and housing cleanliness (clean to very dirty), udder morphology (udder depth, udder balance, height of rear attachment), milking practices (udder washing before milking, usage of soap, udder wiping after washing, forestripping, post teat dipping, feeding cow after milking), and milking machines (liner status, tubes status, claw status, cleanliness of milking machine). All variables with p-value < 0.2 in the initial univariable analysis were checked for collinearity ($r \geq 0.60$) using Spearman rank correlation. Variables which did not show collinearity were considered in a multivariable logistic regression analysis. In addition, if two variables showed collinearity ($r \geq 0.60$), the one with the lowest p-value was also introduced in this model. In this analysis, statistical significance was set at $p < 0.05$.

Results

Prevalence of bovine mastitis and somatic cell count distributions

Prevalence of mastitis using clinical observations and CMT tests

The global prevalence of bovine mastitis was 60.3%. Out of this, 48.7% were subclinical mastitis and 11.6% were clinical mastitis cases as determined by CMT and clinical examinations of udder (Table 1). Examining CMT test scores at quarter level, from a total of 1068 quarters, 98.9% are functional. From these functional quarters tested (1056), 30.1% were found to be positive for mastitis where 3.7% were clinical mastitis (swollen and painful quarter or presence of clots and blood in milk) and 26.4% were subclinical cases (CMT score ≥ 2), with a minority of high infection levels (scores 3 and 4, respectively, in 7.9% and 1% of functional quarters; Table 2).

Individual milk somatic cell concentrations

The arithmetic mean of ISCC was $1083 \times 10^3 \pm 2987.2 \times 10^3$ cells/mL (SCS = 4.1 ± 2.8), all numbers and stages of lactations combined. According to the rules stated by Noireterre (2006), Fig. 1 shows a great variability of ISCC between the dairy cows studied, 43.8% of cows had ISCC less than 200×10^3 cells/mL (Healthy cows), 22.1% between 200×10^3 and 500×10^3 cells/mL, and 22.9% of cows had ISCC greater than 1000×10^3 cells/mL.

Table 1 The prevalence of sub-clinical and clinical mastitis at cow level

Results	Cows tested	Prevalence of CM based on clinical signs	Prevalence of SCM based on CMT	Total
Number of cows	267	31	130	161
Percentage (%)	100	11.6	48.7	60.3

CM clinical mastitis, *SCM* sub-clinical mastitis

Table 2 The prevalence of sub-clinical and clinical mastitis at quarter level

Results		Number of quarters	Percentage (%)
Healthy quarters	Score 0	540	51.1
	Score 1	198	18.8
Total		738	69.9
Positive functional quarters	CMT score ≥ 2	279	26.4
	Score 2	185	17.5
	Score 3	83	7.9
	Score 4	11	1.0
	Presence of clinical signs (swollen and painful quarter/presence of clots and blood in milk)	39	3.7
Total		318	30.1
Total functional quarters		1056	100

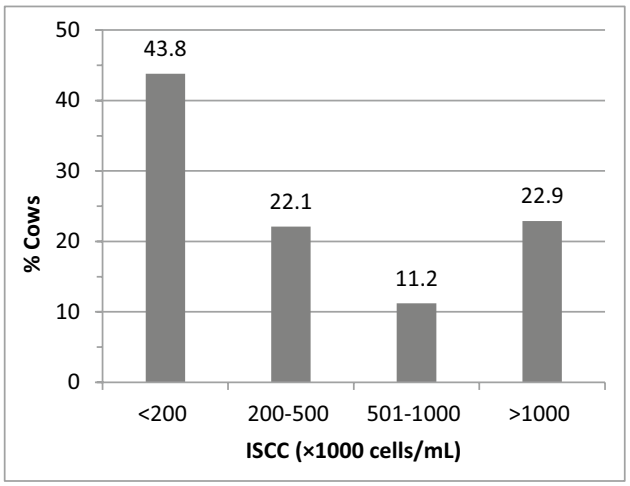

Fig. 1 ISCC distribution among cows

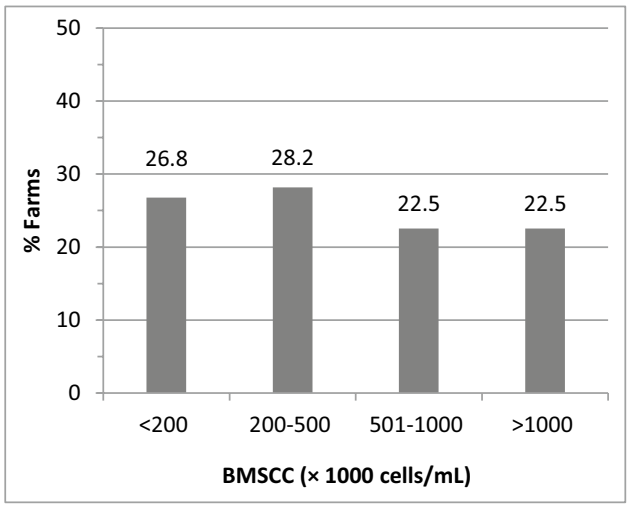

Fig. 2 BMSCC distribution among studied farms

Bulk milk somatic cell concentration

The median and mean BMSCC for all herds visited were 437×10^3 cells/mL and $698 \times 10^3 \pm 726.3 \times 10^3$ cells/mL (SCS = 4.9 ± 1.9), respectively, all ranks and stages of lactations combined, which exceeded the Tunisian standard (NT-14–141-2004) for industrial acceptance of milk (500 10^3 cells/mL). The distribution of BMSCC showed that 45% of farms had BMSCC greater than 500×10^3 cells/mL (Fig. 2). BMSCC mean is associated with high standard deviations, which indicate heterogeneity of the quality of the milk produced in the different farms. BMSCC varied between the two regions studied with 683.7×10^3 cells/mL and 709.1×10^3 cells/mL, for north and center east regions, respectively (Fig. 3).

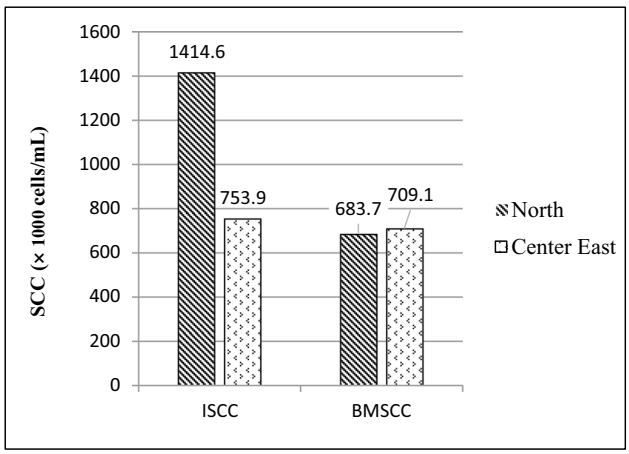

Fig. 3 Mean ISCC and BMSCC between regions

Risk factors

Among risk factors considered for univariable logistic regression for the presence of mastitis, lactation stage,

parity, udder depth, individual cow cleanliness, liner status, tubes status, and cleanliness of milking machine were significantly ($P < 0.05$) associated with mastitis prevalence. In addition, udder balance, type of milking, usage of soap for udder washing, and claw status had a P-value < 0.2 (Table 3 and 4).

The final multivariable logistic regression model revealed that among herd and cow-level risk factors studied in this model, parity, lactation stage, udder depth, and type of milking had a significant effect ($P < 0.05$) on mastitis prevalence (Table 5). Accordingly, the likelihood of mastitis prevalence was 1.9 times higher in multiparous cows compared with primiparous cows. Similarly, cows with an udder floor below and at the level of the hock had 3.3 and 2.1 times respectively more chance to have mastitis than cows with an udder floor above the hock.

The occurrence of mastitis was 4.6 and 2.2 times more likely in cows in the late lactation and mid lactation respectively compared to cows in the early lactation. Cows that are mechanically milked were 3.8 more likely to get mastitis than cows that are manually milked.

However, udder balance, individual cow cleanliness, usage of soap, and milking machine characteristics had no significant effect ($P > 0.05$). But there was a tendency of cow individual cleanliness to increase udder inflammation when cows were classified as more and more dirty (OR = 2.7; $p = 0.067$).

Etiology of bovine mastitis

Microbiological analysis indicated that 83.8% (109/130) of all CMT-positive samples (CMT ≥ 2) had a detectable bacterial infection. However, 16.2% were bacteriologically negative in our conditions. The identification of bacteria revealed the presence of *Staphylococcus aureus* (13.1%), coagulase negative staphylococci (26.2%), *Escherichia coli* (2.3%), *Streptococcus uberis* (0.8%), *Aerococcus viridans* (6.9%), and other minor germs in lower proportions. Mixed infection was observed in 23.1% of the samples. For clinical mastitis and among 31 samples, 87.1% were positive for bacterial cultures and 12.9% were negative. The isolated bacteria were *Staphylococcus aureus* (32.3%), coagulase negative Staphylococci (19.4%), *Streptococcus uberis* (3.2%), and other minor germs in lower proportions. In addition, 19.4% of isolates had an association between two germs (Fig. 4).

Discussion

Our study showed a very high cow-level mastitis prevalence of 60.3% as determined by the CMT and clinical examinations of the udder. These results are similar to those found in Ethiopia, where 63.02% of cows were affected by mastitis (Lakew et al. 2019). However, the prevalence of mastitis in Tunisia is higher than in other countries. For instance, it was 44.8% in Algeria (Bouzid

Table 3 Descriptive statistics and results from univariable logistic regression analysis between individual cow-level risk factors and Mastitis prevalence

Variable	Level	Cow number *N*	Mastitis cows	Prevalence (%)	*P* value
Breed	Holstein	188	114	60.6	0.976
	Brown Suisse	29	16	55.2	
	Cross breed	46	29	63	
	Others	4	2	50	
Lactation stage	Early lactation	60	27	45	**< 0.001**
	Mid lactation	116	66	56.9	
	Late lactation	91	68	74.7	
Parity	Primiparous	83	35	42.2	**< 0.001**
	Multiparous	184	126	68.5	
Udder depth	Udder floor above the Hock	118	56	47.5	**0.004**
	Udder floor below the Hock	52	41	78.9	
	Udder floor at the level of the Hock	97	64	66	
Udder balance (right vs left)	Balanced udder	160	90	56.3	**0.099**
	unbalanced udder	107	71	66.4	
Height of the rear attachment	< 20 cm	70	40	57.1	0.641
	20–25 cm	172	106	61.6	
	> 25 cm	25	15	60	

Others: breeds included Montbéliarde and Tarentaise

Table 4 Descriptive statistics and results from univariable logistic regression analysis of associations between different risk factors and cow-level mastitis

Variables	Category	Cow number	Mastitis cows	Prevalence (%)	*P* value
Region	North	133	84	63.2	0.342
	Center-East	134	77	57.5	
Production system	Semi intensive	133	84	63.2	0.342
	Above-ground	134	77	57.5	
Type of milking	Manual	26	11	42.3	**0.053**
	Mechanical	241	150	62.2	
Udder washing before milking	Teat only	154	95	61.7	0.588
	Whole udder	113	66	58.4	
Usage of soap	Water only	198	124	62.6	**0.189**
	Water + soap	69	37	53.6	
Udder wiping after washing	Yes (collective wet cloths)	78	49	62.8	0.589
	No	189	112	59.3	
Forestripping	Always	12	8	66.7	0.986
	Never	150	89	59.3	
	Sometimes	105	64	61	
Post-teat dipping	Yes	64	40	62.5	0.680
	No	203	121	59.6	
Feeding cows after milking	Yes	166	100	60.2	0.980
	No	101	61	60.4	
Individual cow cleanliness	Clean	38	18	47.4	**0.044**
	Slightly dirty	120	72	60	
	Dirty	70	43	61.4	
	Very dirty	39	28	71.8	
Housing cleanliness	Clean	113	65	57.5	0.277
	Slightly dirty	83	51	61.5	
	Dirty	48	28	58.3	
	Very dirty	23	17	73.9	
Liner	Unsatisfactory	119	80	67.2	**0.014**
	Satisfactory	122	70	57.4	
Tubes	Unsatisfactory	96	65	67.7	**0.019**
	Satisfactory	145	85	58.6	
Claw	Bad	125	78	62.4	**0.164**
	Good	116	72	62.1	
Cleanliness of milking machine	Bad	209	133	63.6	**0.025**
	Good	32	17	53.1	

et al. 2011) and 42.9% in Egypt (Elbably et al. 2013). In India, the overall prevalence of bovine mastitis was 41.9% (Tripathy et al. 2018), and in Bangladesh, it was 28.6% (Hoque et al. 2018). The variation in mastitis prevalence from one country to another and even within the same country can be attributed to the breed, milking, and hygiene practices applied in each farm, to the different preventive measures used, and to the animal (Radostitis et al. 2007). Moreover, it may be due to the different sensitivities of method of mastitis screening used to manage udder health.

The prevalence of clinical and subclinical mastitis was 11.6% and 48.7%, respectively. The clinical prevalence in this study was similar to the report of 12.5% by Zeryehun and Abera (2017) in Ethiopia and higher than 6.8% reported by Lakew et al. (2019) in the same country and Mbindyo et al. (2020) in Kenya. In the case of subclinical mastitis, the prevalence at cow level (48.7%) in this study was in agreement with the finding 48.8% reported by Suleiman et al. (2018) in Tanzania and 52% by Iraguha et al. (2015) in Rwanda but higher than 28.6% reported by Saidi et al. (2013) in Algeria and 20.5% reported by Olivares-Pérez

Table 5 Multivariable logistic regression analysis of association between different risk factors and cow-level mastitis in the North and the Center East regions of Tunisia

Variable	Category	OR	95% CI for OR	*P* value
Parity	Primiparous	Ref		
	Multiparous	1.948	1.008–3.764	**0.047**
Lactation stage	Early lactation	Ref		
	Mid lactation	2.253	1.105–4.593	**0.025**
	Late lactation	4.632	2.078–10.328	**< 0.001**
Udder depth	Udder floor above the Hock	Ref		
	Udder floor below the Hock	3.336	1.386–8.030	**0.007**
	Udder floor at the level of the Hock	2.100	1.063–4.149	**0.033**
Udder balance (right vs left)	Balanced udder	Ref		
	Unbalanced udder	1.095	0.601–1.994	0.767
Individual cow cleanliness	Clean	Ref		
	Slightly dirty	1.587	0.712–3.539	0.259
	Dirty	1.782	0.738–4.306	0.199
	Very dirty	2.724	0.931–7.974	0.067
Usage of soap	Water only	Ref		
	Water + soap	0.812	0.423–1.558	0.531
Type of milking	Manual	Ref		
	Mechanical	3.767	1.374–10.331	**0.010**
Liner	Unsatisfactory	Ref		
	Satisfactory	0.698	0.290–1.680	0.422
Tubes	Unsatisfactory	Ref		
	Satisfactory	0.753	0.312–1.816	0.528
Claw	Bad	Ref		
	Good	1.506	0.781–2.905	0.221
Cleanliness of milking machine	Bad	Ref		
	Good	0.431	0.178–1.041	0.061
R^2		**24.8%**		

Factors statistically significant at $p \leq 0.05$

OR, odds ratio; *CI*, confidence interval; *Ref*, reference category; R^2, coefficient of determination

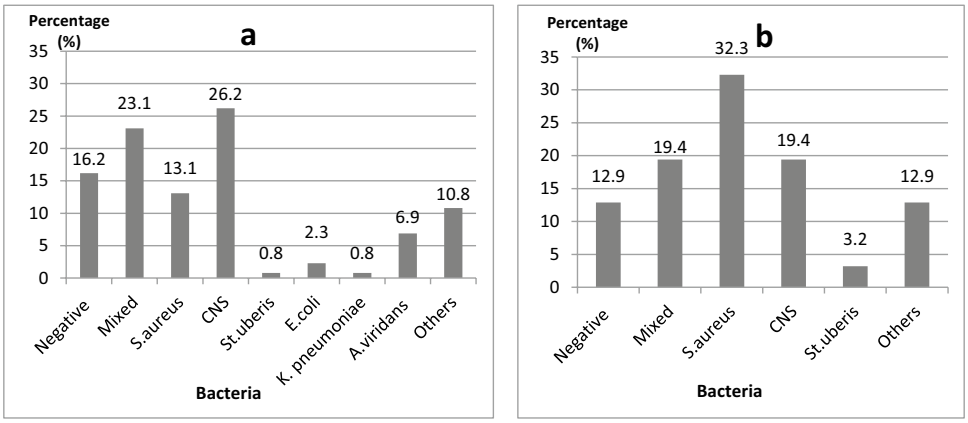

Fig. 4 Percentage of different germs isolated from subclinical (**a**) and clinical (**a**) mastitis samples. Mixed, association between two germs; *S. aureus*, *Staphylococcus aureus*; *CNS*, coagulase-negative Staphylococci; *St. uberis*, *Streptococcus uberis*; *E. coli*, *Escherichia coli*; *K. pneumoniae*, *Klebsiella pneumoniae*; *A. viridans*, *Aerococcus viridians*

et al. (2015) in Mexico. According to our study and the results of other authors, we noticed that the frequency of subclinical mastitis remains higher than that of the clinical one. This difference could be explained by the lack of attention given to subclinical mastitis when treating clinical cases (Lakew et al. 2019) or to the udder defense mechanism which serves to reduce the severity of the disease (Erskine 2001; Sori et al. 2005) and make it undetected. This form of mastitis, when treated, also leads to increased antimicrobial resistance observed with most pathogens causing mastitis

(Motaung et al. 2017). In Africa, the high prevalence of mastitis might be attributed to the lack of udder health program and of regular cattle screening for subclinical and clinical signs of mastitis by farmers in order to treat cows timely. Milk quality policies and independent organized control quality protocols to allow differential payment of milk, which are very incentive to improve management practices and reduce milk bacteria concentrations and SCC of milk (Botaro et al. 2013; Pašić et al. 2016), are also lacking.

Our findings showed a high mean of ISCC and BMSCC and globally bad milk quality in the studied regions as confirmed in many previous studies in Tunisia (Ben-Salem et al. 2006; Kamoun 2011; Bouraoui et al. 2014; Gargouri et al. 2014; M'Sadak et al. 2015; Darej et al. 2019). Above 400×10^3 cells/mL, the possibility of the cow being affected by a major pathogen is high (Hanzen 2008). A bacterial infection can cause a severe and acute ISCC increase greater than 10^6 cells/mL (Bytyqi et al. 2010). This shows the high number of individual cows with untreated mastitis in each herd studied. The high BMSCC was situated above the international standard (milk collection is stopped when exceeding a threshold of 750 000 cells/mL in the USA, 500 000 cells/mL in Canada, 400 000 cells/mL in Europe, New Zealand, and Australia, 350 000 cells/mL in Switzerland; Sharma et al. 2011) and implied that the milk collected is dangerous for human health (especially when crude milk is used) and has poor technological abilities (Bobbo et al. 2016).

Among the individual risk factors selected in multivariable model in our study, parity had a significant effect on the prevalence of mastitis. The relative risk of mastitis was higher in multiparous cows compared with primiparous cows. These results agree with a Tunisian study by M'sadak et al. (2016) and many others throughout the world (Biffa et al. 2005; Parker et al. 2007; Nyman et al. 2009; Mekibib et al. 2010; Taponen et al. 2017; Ndahetuye et al. 2019). The increase in mastitis prevalence in multiparous cows was explained by many factors as first, the higher udder depth (also significant in our study), and increased teat length that bring teats closer to the floor and increase the risk of teat injuries when the cow is lying down (Singh et al. 2014; Sharma et al. 2016) and of contamination by environmental pathogens (Bhutto et al. 2010; Nakov et al. 2014). Second, aging increases loss of elasticity of the sphincter with relaxation of the muscles and increases its permeability which facilitates penetration of pathogens (Radostitis et al. 2007; Suleiman et al. 2018). But another reason is the persistent infections over years due to pathogens like *Staphylococcus aureus* which have long-term persistence in mammary gland due to their ability to form biofilms in ducts and alveoli and to invade and/or survive intracellularly even after an intramammary antibiotic treatment (Grunert et al. 2018). This germ, able to persist in gland in equilibrium with immune defense, is known as one of the main contributors to subclinical mastitis (Birhanu et al. 2017). In addition, cows in early lactation had significantly less mastitis than cows in the mid and late lactation stage, as previously shown by other authors (Mureithi and Njuguna 2016) for mid lactation stage and (Belayneh et al. 2013; Tolosa et al. 2013; Abrahmsén et al. 2014; Ndahetuye et al. 2020) for late stage. Repeated exposure of cow to milking process could explain increasing risks of contagious mastitis at the end of lactation (Almaw et al. 2008; Mekonnen et al. 2017). This is especially important in Tunisia where we showed that cows mechanically milked had 3.8 more chances to get mastitis than cows manually milked. Indeed, bad machine settings can modify significantly the milking efficiency and milk quality, increase the risk of udder aggression, and decrease udder health status (Marnet 2013). More specifically, irregular vacuum fluctuations can have an impact on udder health and teat condition and can lead to increased infection rates (Mein 2012; Besier et al. 2016). However, the multivariable model did not show any significant effect of the general status of milking equipment (liners, tubes, and claw) and of the milking machine cleanliness on the mastitis prevalence. These results are not in agreement with M'Sadak et al. (2013) who showed that the characteristics of the milking machine (cleaning of the milking machine, condition of the pipes) present a significant influence on individual somatic cell counts (ISCC). Maybe, our visual scoring of the milking machine was not sufficiently discriminant to explain this increase of mastitis prevalence. But another reason could be that mastitis in these herds had an infectious origin (between animals) rather than an environmental origin (from environment to animals; Bosquet et al. 2013; Bharti et al. 2017) as suggested by the very high SCC that we have recorded (largely over 300×10^3 cells/mL). In this case, an assessment of the vacuum and pulsation levels and of proper operation control of the different components of machine would be essential to confirm a very probable problem at machine level operation.

For the hygienic practices, usage of soap had no significant effect on mastitis prevalence. These results may be due to a poor practice quality. The water used for washing the udder whatever alone or with soap was used for all the cows of each herd.

Statistically, the very dirty cows had only a tendency to increase mastitis prevalence than clean cows that could be due to the high variability of conditions we found between farms and not sufficiently discriminant method of scoring used. This tendency is confirmed in other studies (Schreiner and Ruegg 2003; Rahman et al. 2009; Iraguha et al. 2015). Nevertheless, our main result is that cows, in this specialized dairy zone of Tunisia, were globally dirty in the majority of farms. Since cleanliness is also an important indicator of

cow welfare (Hultgren and Bergsten 2001; Ellis et al. 2007) that could have a twofold effect on productivity, it has to be fixed as soon as possible.

Knowledge of the udder pathogens causing mastitis remains very important for correct orientation of proactive programs and monitoring of udder health in dairy herds (Schukken et al. 2003; Ruegg 2011). The present study showed that 83.8% of subclinical mastitis and 87.1% of clinical mastitis are of bacterial origin. Among the bacteria isolated, Gram-positive cocci strains were the most frequent, notably 32.3% of *S. aureus* and 19.4% of CNS in the clinical mastitis samples and 13.1% of *S. aureus* and 26.2% of CNS in the subclinical mastitis samples. In addition, in the case of mixed infection, most of the associations were with one of these two germs. These results agree with many studies (Canada, the USA, France, Ethiopia, China) which confirmed that *S. aureus* and CNS are the most frequently isolated pathogens in subclinical or clinical mastitis of infectious origin (Olde Riekerink et al. 2008; Schukken et al. 2009; Botrel et al. 2010; Mekonnen et al. 2017; Sun et al. 2017). In Tunisia, our results are similar to a previous study conducted in the north area, which recorded that CNS (79.7%) are the main bacteria responsible for mastitis (Ben Hassen et al. 2003). In Algeria too, results agree with our finding and showed that *S. aureus* (Saidi et al. 2013) and CNS (Zaatout et al. 2019) were the most widespread agents of subclinical mastitis. Likewise, in Egypt, *S. aureus* was most frequently isolated (52.5%) in cases of subclinical mastitis (Abdel-Rady and Sayed 2009). We did not confirm the recent studies in Tunisia which showed a high prevalence of *E. coli* and *Klebsiella* spp., two strains mainly associated to environmental etiology in clinical bovine mastitis (Saidani et al. 2018; Klibi et al. 2019). The specificity of the farms studied by these authors, the study area, and season could explain this discrepancy.

S. aureus has, as main reservoir, the skin of the udder and the milk of the infected udder. Such a high frequency of staphylococcal mastitis in our study confirmed the contagious classification of mastitis and the udder and milking origin of infection aggravated by an insufficient milking hygiene (animal and machine) as this species spread during the milking process from cow to cow (Harmon 1994).

Although staphylococci represent the main pathogens involved in subclinical and clinical mastitis, other bacterial species have been found but with lower proportions such as *St. uberis*, *E. coli*, *A. viridans*, *Micrococcus* spp., *Pasteurela* spp., *Pseudomonas luteola*, and *Burkholderia cepacia*. For the prevalence of mastitis, the frequency and origin of udder infection vary from country to country. This is due to the difference in preventive measures and milking procedures on each farm (Bradley 2002; Haltia et al. 2006), different management factors, (Green et al. 2007), milk leakage, and previous udder infection (Mungube et al. 2005). For these reasons, data on the frequencies of germs causing bovine mastitis should be considered with caution. About 23.1% of cases of subclinical mastitis and 19.4% of clinical mastitis presented a mixed infection at the udder level (for which two bacteria were responsible). This high rate of mixed infection might be more likely due to the variety of infection between quarters (the analysis was done on composite milk of 4 quarters) than to the possible contamination of samples.

In conclusion, the north and center east regions of Tunisia are important dairy basins. The bovine mastitis prevalence in these regions was very high. This may reveal the economic loss suffered by the dairy sector. The effect of udder morphology on the mastitis prevalence requires further studies in Tunisian conditions in order to find solutions to help the reduction of mastitis in farms (genetic male selection or female culling policy). Mastitis was mainly of contagious origin with the prevalence of *S. aureus* and CNS. This justifies a feasible intervention strategy against mastitis, with particular emphasis on contagious mastitis, hygienic milking practices, and machine hygiene. Further studies are needed to confirm probable impact of machine milking operation. Therefore, one of the best ways to encourage breeders to perform good practices and right prevention program, in order to reduce the incidence of mastitis in Tunisian breeding, is to reward breeders for their production of clean milk by paying milk for quality.

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Author contribution All authors contributed to this study. A.M. and A.T. designed the study. A.M., H.M., and N.S conducted the experimental work. A.T. and P.G.M supervised the study. A.M and F.S. analyzed the data. A.M., A.T., and P.G.M. wrote, reviewed, and edited the manuscript. All authors read and approved the final manuscript.

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Data availability Data will be made available on reasonable request.

Declarations

Ethics approval Institutional animal ethics guidelines were followed for the experiment. Studied animals were subjected to minimum stress during milk sampling.

Conflict of interest The authors declare no competing interests.

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