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Product Quality and Safety

Bacteria variety causing clinical mastitis in Holstein-Friesian cows in Pelagonia region, North Macedonia

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Abstract. *The main aim of this study are the bacteria that most often cause clinical mastitis (CM) and their impact on milk reduction in Holstein-Friesian cows in the Pelagonia - North Macedonia region. 36 milk samples were taken from Holstein-Friesian breed of cows with confirmed clinical mastitis by a veterinarian. The samples were taken for the period from January 2019 to December 2020 from 20 different smallholder farms situated in the monitored region. Two sterile tubes with 10 ml of milk in each of them were taken from the affected part of the udder of the cow. A total of 86 tubes with milk from 36 mastitis cows were taken. From each sample 300 µl drips were placed in petri dishes with different selective nutrient media: Mannitol Salt Agar, MacConkey Agar, Endo Agar and Edwards nutrient medium. The petri dishes were incubated at 35±2°C for 24-48 hours in Mannitol Salt Agar, at 30-35°C for 18 to 72 hours in MacConkey Agar, at 35±2°C for 18 to 24 hours in Endo Agar and at 35-37°C for 24-48 hours in Edwards nutrient medium. Morphology of colonies and cells were examined with a microscope. A total of 119 strains were obtained and the following physiological and biochemical studies were performed to determine the new isolates: oxidase reaction, catalysis activity, indol test, hydrolysis of the hyporate, acetoin formation (acetylmethylcarbinol, Voges-Proscauer reaction) and Methyl-Roth test (MR- test). The results obtained revealed that the most common bacterial species causing clinical mastitis in Holstein-Friesian cows in 2019 and 2020 were six species of bacteria, where E. coli and Staphylococcus spp. are dominant.*

Keywords: *E. coli*, *Staphylococcus* spp., *Streptococcus* spp., *Klebsiella* spp., *Pasteurella* spp., *Proteus* spp.

Introduction

Clinical mastitis is defined as inflammation of the mammary glands or udder in cows. This inflammation can be subclinical where there are no visible signs of infection, clinical with signs of infection or chronic when symptoms persist for a longer period of time. Clinical mastitis causes great losses in the dairy industry, respectively on the farm economic efficiency, affects animal health by losing the functional quarter, reduced milk production and in rare cases it can be fatal (Burvenich et al., 2003; Gröhn et al., 2005; Hertl et al., 2011; Ibrahim, 2017; Stankov, 2020). The peak of clinical mastitis occurs at the time of delivery or immediately before and after it (Hagnestam-Nielsen and Østergaard, 2009; Fitzpatrick et al., 2013). Infections can easily occur in the area of the udder (Coulona et al., 2002; Merin et al., 2008; Barkema et al., 2009). Attention should be paid to the hygiene of the mammary gland because there are large number of different bacteria on it and in the environment (Hagi et al., 2013; Bekuma and Galmessa, 2018; Zigo et al., 2021). Infection occurs when pathogenic microorganisms enter the mammary gland through the mammary duct (Zhao and Lacasse, 2008; Akers and Nickerson, 2011; Zigo et al., 2021). According to Bannerman (2009), the specific inflammatory response from mastitis depends on the bacterial species involved. From this point of view, the studies determining which pathogens have the greatest impact on cow health, production, and profitability are valuable for the adequate treatment to be applied.

During milking the appearance of somatic cells and their number in the milk should be noted regularly (Bradley and Green, 2005; Nyman et al., 2007; Alhussien and Dang, 2018). If after 10 days of lactation the number of somatic cells exceeds 150,000 in 1 ml of milk, this is a sign that already about 5% of the cows in the herd have a problem with the udder (Barkema et al., 1998; Bradley and Green, 2005, Santman-Berends et al., 2012. Hiitiö et al., 2017).

Bacteria that cause clinical mastitis can be divided into three groups. In the first group are gram-positive bacteria - *Streptococcus* spp. and *Staphylococcus* spp. The second group includes gram-negative bacteria - *Escherichia coli*,

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Klebsiella spp., *Citrobacter* spp and *Enterobacter* spp. The last group includes *Mycoplasma bacteria* spp., *Corynebacterium* spp. and *Pseudomonas* spp. The sooner clinical mastitis is diagnosed, the sooner the problem can be resolved (Duarte et al., 2015; Ashraf and Imran, 2018). All cows should be treated with antibiotics regardless of whether they have clinical mastitis. This will prevent further spread. When successful prevention of mastitis is carried out, treatment of animals is not required (Barkema et al., 2006; Klocke et al., 2010). Cows should be provided with the best possible hygiene conditions (De Palo et al., 2006; Conte and Scarantino, 2013) to prevent the occurrence of clinical mastitis.

The purpose of this study was to determine the bacteria that most often cause clinical mastitis in Holstein-Friesian cows in Pelagonia region, North Macedonia. The obtained data would allow to develop adequate measures to limit this disease in highly productive cows in the country.

Material and methods

Object of study

The objects of study were Holstein-Friesian cows with confirmed clinical mastitis by a veterinarian. The animals were reared in 20 different smallholder farms in Pelagonia region, North Macedonia. The number of raised cows varied from 10 to 30 cows per farm. In all farms a semi-open system of the housing of the cows was used. The cows stay on the farm all the time. In the buildings, the cows are reared tied on concrete beds (2.20-2.40/1.05-1.20 m) with straw litter. During the day the cows are untied to move freely in the yard in front of the relevant building. The manure and other wastes from the facilities were mechanically cleaned twice a day.

The average milk production of the cows from all 20 farms for 305 days' lactation period was 6000 to 7500 kg. The cows were milked two times a day (morning and evening) while tied on the beds by a vacuum milking system. The system is supplied with milk cups which are connected with a vacuum system of pipes carrying the milk directly in a galvanized container or tank (milk freezer).

The cows were fed with rations according to the season and the provided fodder. The winter diet was combined with corn silage - 25 kg, alfalfa hay - 5 kg, dry slices of sugar beet - 2 kg, dry by-product of beer - 2 kg and concentrate as a mixture – 8 kg. The summer diet was combined with corn silage -12 kg, green alfalfa - 10 kg, green oilseed rape - 8 kg, alfalfa hay - 1 kg, dry slices sugar beet – 2.2 kg and concentrate as a mixture - 7.5 kg.

Samples

A total of 36 milk samples were taken from 36 cows with confirmed clinical mastitis by a veterinarian from all 20 farms in the monitored region for the period January 2019 - December 2020 (Table 1). Two sterile tubes with 10 ml of milk in each of them were taken from the affected part of the udder of the cow. A total of 86 tubes of milk samples from all 36 mastitis cows were taken.

Growth of selective nutrient media

From each sample taken 300 µl of the milk was dropped in petri dishes with different selective nutrient media. The seeded petri dishes were incubated at 35±2°C for 24-48 hours in Mannitol Salt Agar - Sigma-Aldrich, USA, at 30-35°C for 18 to 72 hours in MacConkey Agar - Thermo Fisher Scientific, USA, at 35±2°C for 18 to 24 hours in Endo Agar - Sigma-Aldrich, USA and at 35-37°C for 24-48 hours in Edwards nutrient medium - Thermo Fisher Scientific, USA. The selective nutrient media that we used were prepared following the manufacturer's instructions.

Morphology of colonies and cells

Classical microbiological methods were used to determine the morphology of colonies and cells (Nebe-von-Caron et al., 2000). A light microscope was used to determine the morphology of the columns and the cells of the bacteria.

Isolation of bacteria

A total of 119 strains were isolated (Table 2).

Physiological and biochemical parameters of the bacteria isolated

We performed the following physiological and biochemical studies to determine the new isolates:

Catalysis activity: Material of the tested strain was incubated in a drop of 3% H_2O_2 for up to 5 minutes. The positive result was reported by formation of bubbles and the negative - by their absence.

The oxidase reaction: The tested strain was placed on a slide and then incubated with reagents. First, a solution of diethyl (or tetramethyl) para-phenylenediamine HCl in distilled water with concentration 10 mg/ml was prepared. Then a drop of para-phenylenediamine was applied on the slide. If there was appearance of dark purple-red discoloration after 10 seconds, the reaction was positive and if there was appearance of a pale yellow color, the reaction was negative.

Indol test: For the test a nutrient medium (pH 7.2-7.4) made by the following components was used: bacto-peptone 10 g; NaCl 5 g and distilled water 100 ml. The prepared nutrient medium was placed in an autoclave at 121°C for 20 min and placed in tubes of 9 ml. The strains were placed in test tubes and cultured for 24 and 48 hours at 37°C. Kovac reagent was made from p-dimethylaminobenzaldehyde 5 g and Isoamyl alcohol 75 ml. 0.2 ml of Kovac`s reagent were added to the culture and incubated for 24 or 48 hours. Then the test tubes were resuspended. We registered positive reaction when there was a dark red color on the reagent layer and a negative reaction when the reagent layer remained light yellow.

Hydrolysis of the hyporate: First, we prepared a liquid medium with 1% sodium hypurate. The food base was placed in 8 ml tubes. Then 12 g of $FeCl_3$ were dissolved in 2% HCl to the final volume of 100 ml prepared and after that 0.2 ml of the solution was added to the test tubes. The appearance of precipitates within 10-15 minutes we registered as positive reaction and the absence of precipitation - as negative.

Acetoin formation (acetylmethylcarbinol, Voges-Proscauer reaction): For that purpose a Methyl Red Voges Proskauer Broth (MR-VP broth) medium with the following

components was used: 7 g Bacto peptone, 5 g K_2HPO_4, 5 g Glucose and 1000 ml distilled water, pH 7.5. The nutrient medium was separated in 2-3 ml tubes and sterilized at 0.5 atm for 20 min. Two reagents were prepared - A and B. Reagent A was made with 5 g α-naphthol dissolved in 100 ml absolute ethanol. The color of the reagent should be pale yellow but not darker. Reagent B was made with 40 g CON dissolved in 100 ml distilled water. Glass tubes with WP medium were inoculated with the examined cultures. Then the tubes were incubated for 4 days at 37°C. After the incubation in each test tube, 0.6 ml of reagent A and 0.2 ml of reagent B was added. If the solution was colored in red, the reaction was registered as positive and if there was no color or if, it was a pale pink - the reaction was negative.

Methyl-Roth test (MR - test): For the test a MR-VP broth medium was made with the following components: 7 g Bacto peptone, 5 g K_2HPO_4, 5 g Glucose and 1000 ml distilled water, pH 7.5. The nutrient medium was separated in 2-3 ml tubes and sterilized at 0.5 atm for 20 min. MR solution was made with 0.25 g methyl red dissolved in 100 ml 75% ethanol. In the tubes with MR-VP medium the examined cultures were added. Then the tubes were cultured for 4 days at 37°C. After the incubation one drop of the MR solution was added to each test tube. Appearance of red color shows a positive reaction and the appearance of yellow color shows negative reaction.

Table 1. Milk samples taken from the Holstein-Friesian cows with clinical mastitis (double quarters and single quarter), 2019-2020

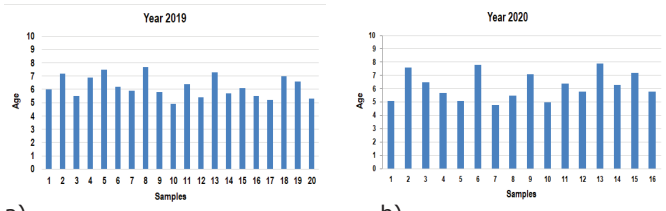
Sample	Year	Age	Milk taken from a of the udder		Number of tubes taken
			Double quarters	Single quarter	
1	2019	6.0	X	-	4
2	2019	7.2	-	X	2
3	2019	5.5	-	X	2
4	2019	6.9	-	X	2
5	2019	7.5	-	-	4
6	2019	6.2	-	X	2
7	2019	5.9	-	X	2
8	2019	7.7	X	-	4
9	2019	5.8	-	X	2
10	2019	4.9	-	X	2
11	2019	6.4	-	X	2
12	2019	5.4	-	X	2
13	2019	7.3	X	-	4
14	2019	5.7	-	X	2
15	2019	5.7	-	X	2
16	2019	6.1	-	X	2
17	2019	5.5	-	X	2
18	2019	5.2	-	X	2
19	2019	7.0	X	-	4
20	2019	6.6	-	X	2
21	2020	5.3	-	X	2
22	2020	5.1	-	X	2
23	2020	7.6	-	X	2
24	2020	6.5	-	X	2
25	2020	5.7	-	X	2
26	2020	5.1	-	X	2
27	2020	7.8	X	-	4
28	2020	4.8	-	X	2
29	2020	5.5	-	X	2
30	2020	7.1	X	-	4
31	2020	5.0	-	X	2
32	2020	6.4	-	X	2
33	2020	5.8	-	X	2
34	2020	7.9	X	-	4
35	2020	6.3	-	X	2
36	2020	7.2	-	X	2

Table 2. Isolates obtained from samples taken from clinical mastitis Holstein-Friesian cows in the Pelagonia region for 2019 and 2020

No	Strains	No	Strains	No	Strains	No	Strains	No	Strains	No	Strains	No	Strains
1	01-M19	18	18-M19	35	35-M19	52	52-M19	69	69-K20	86	86-K20	103	103-K20
2	02-M19	19	19-M19	36	36-M19	53	53-M19	70	70-K20	87	87-K20	104	104-K20
3	03-M19	20	20-M19	37	37-M19	54	54-M19	71	71-K20	88	88-K20	105	105-K20
4	04-M19	21	21-M19	38	38-M19	55	55-M19	72	72-K20	89	89-K20	106	106-K20
5	05-M19	22	22-M19	39	39-M19	56	56-K20	73	73-K20	90	90-K20	107	107-K20
6	06-M19	23	23-M19	40	40-M19	57	57-K20	74	74-K20	91	91-K20	108	108-K20
7	07-M19	24	24-M19	41	41-M19	58	58-K20	75	75-K20	92	92-K20	109	109-K20
8	08-M19	25	25-M19	42	42-M19	59	59-K20	76	76-K20	93	93-K20	110	110-K20
9	09-M19	26	26-M19	43	43-M19	60	60-K20	77	77-K20	94	94-K20	111	111-K20
10	10-M19	27	27-M19	44	44-M19	61	61-K20	78	78-K20	95	95-K20	112	112-K20
11	11-M19	28	28-M19	45	45-M19	62	62-K20	79	79-K20	96	96-K20	113	113-K20
12	12-M19	29	29-M19	46	46-M19	63	63-K20	80	80-K20	97	97-K20	114	114-K20
13	13-M19	30	30-M19	47	47-M19	64	64-K20	81	81-K20	98	98-K20	115	115-K20
14	14-M19	31	31-M19	48	48-M19	65	65-K20	82	82-K20	99	99-K20	116	116-K20
15	15-M19	32	32-M19	49	49-M19	66	66-K20	83	83-K20	100	100-K20	117	117-K20
16	16-M19	33	33-M19	50	50-M19	67	67-K20	84	84-K20	101	101-K20	118	118-K20
17	17-M19	34	34-M19	51	51-M19	68	68-K20	85	85-K20	102	102-K20	119	119-K20

Results and discussion

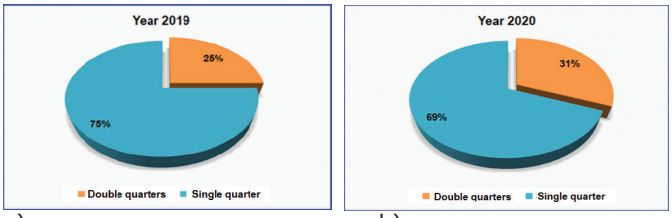
For a period of two years, 36 milk samples of 36 mastitis cows were taken from 20 different farms in the Pelagonija region of North Macedonia. The age of the cows from which the samples were taken is shown on Figure 1. The results in Figure 1a show that the samples in 2019 were taken from cows at different ages – between 4.9 and 7.7 years. The same were the results for 2020 (Figure 1b) - between 4.8 and 7.7 years, respectively. From the results, it can be concluded that in both years of the examination the age of the cows with mastitis demonstrated similar effect. Therefore, the age of the cows in very rare cases can be related with occurrence of clinical mastitis. Khokon et al. (2017) found that the incidence of mastitis was significantly higher in 3.5-4.5-year-old cows and lower in 2.5-3.5-year-old ones. Comparing our results with the results of those authors we can see that according to our examination the occurrence of mastitis in cows is with a higher age limit.



a) b)

Figure 1a,b. The age of Holstein-Friesian cows with clinical mastitis in 2019 and 2020

Clinical mastitis can usually affect part of the udder and in rare cases affects the entire udder of dairy cows. The results in that aspect are presented on Figure 2. The results on Figure 2a shows that in 75% of the cases of mastitis the samples were taken only from the affected part of the udder and in the other 25% - from the whole udder. Figure 2b shows that the samples taken from dairy cows were 69% from the infected area of udder and 31% from the total udder of the cows. From these results it can be concluded that clinical mastitis most often affects only part of the udder of dairy cows. Also, it can be noticed that in both years of our examination there is no increase in the percentage, i.e. that most often the occurrence of clinical mastitis affects 70% of the udder in the hind area. The obtained results are similar with the results of Rahman et al. (2012) who found that clinical mastitis usually affects only a quarter, not 2nd, 3rd or 4th quarters of a cow's udder.

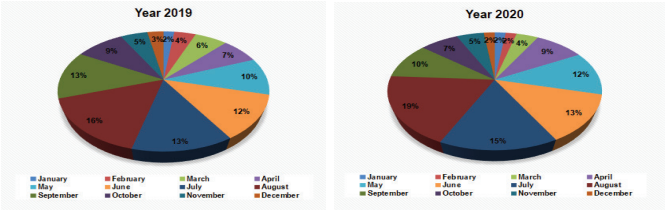


a) b)

Figure 2a,b. Samples taken from the udder for examination in 2019 and 2020 (Single quarter and Double quarters)

The exact month in which the sample is taken has a major relation to the incidence of clinical mastitis. Figures 3a,b show the percentage of mastitis cows in 2019 and 2020 by months. From the results presented it can be seen that in both years of the monitored period the numbers of clinical mastitis increase from March to October, most likely due to the increase of the air temperature. In the period May-September, the cows with clinical mastitis comprised 64% (2019) and 69% (2020) of all confirmed cases of clinical mastitis cows. Our results are in line with the well-known fact that the rise in environmental temperature leads to an increased number of clinical mastitis of the cows as the higher temperatures have positive effect on the growth of bacteria and lead to increasing number of bacteria in the litter, manure and floor surface in the farms. A similar trend of increasing the number of mastitis in cows with increasing ambient temperature has been reported by other authors.

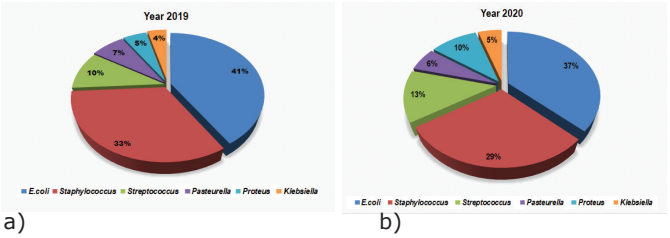
Moris et al. (1988) established that at 3 to 6 years of age cows, monthly incidence of clinical mastitis increased more than 50% above average annual incidence in the hot months as a result of the high monthly air temperature-humidity values. Jingar et al. (2014) found that the increase in temperature-humidity index resulted in increased incidence of mastitis in cows ($p<0.01$) of different breeds. The results obtained, as well as those of other authors (Moris et al., 1988, Shathele, 2009; Jingar et al. 2014), give grounds to assume that the environmental temperature is one of the major preconditions in the occurrence and frequency of mastitis in cows.



a) b)

Figure 3a,b. Distribution (%) of Holstein-Friesian cows with mastitis in 2019 and 2020 by months

In order to make an accurate diagnosis and appropriate treatment, it is necessary to know which bacterium caused the clinical mastitis. The obtained results are presented on Figure 4a,b. It can be seen that the most common species that cause clinical mastitis in Holstein-Friesian cows in Pelagonia region, for 2019 and 2020 are six species of bacteria - *E. coli*, *Staphylococcus* spp., *Streptococcus* spp., *Klebsiella* spp., *Pasteurella* spp. and *Proteus* spp. Of them, *E. coli* and *Staphylococcus* spp. are dominant – with 41 and 37% (2019), and with 33 and 29% (2020), respectively. Our results regarding the percentage distribution of bacteria causing clinical mastitis are similar in some respects and different in others from those of other authors. According to Waage et al. (1999), the most predominant bacteria isolated from bovine mastitis were *Streptococcus* (28.8%), *Escherichia coli* (18.5%), *Streptococcus* (10.8%) and *Staphylococcus* (9.8%), while other bacteria and fungi were isolated in a range of 0.3 to 3.8%. Sumathi et al. (2008) found that the prevalence of the major isolated pathogens from mastitis cows were *Staphylococcus aureus* - 24% and *Escherichia coli* - 20%, followed by *Staphylococcus epidermidis* - 16%, *Streptococcus* spp. - 10% and *Klebsiella* spp. - 10%. Tomazi et al. (2018) established that bacteria most frequently isolated from the samples with clinical mastitis in dairy heifers were *Staphylococcus* (44.3%), *Streptococcus* (18.2%) and *Escherichia coli* (6.4%). The observed diversity in the distribution of the bacteria causing clinical mastitis in cows, from the results of different authors, is normal, as the conditions under which the relevant studies were performed are also different. However, despite the differences in the percentage distribution of bacteria, the presence of the same bacteria species diversity in cows' clinical mastitis occurrence is clearly confirmed.



a) b)

Figure 4a,b. Bacteria causing clinical mastitis in Holstein-Friesian cows in the Pelagonia region for 2019 and 2020

Conclusion

From our conducted examination it was found that: (i) clinical mastitis in Holstein-Friesian cows occurs at the age of 4.8 years to 7.9 years; (ii) over 50% of cows develop mastitis in only a quarter of the udder; (iii) the most common bacteria causing clinical mastitis are *E.coli, Staphylococcus, Streptococcus, Pasteurella, Proteus* and *Klebsiella,* where *E.coli* and *Staphylococcus spp*. are dominant – 37-41% and 29-33%, respectively; (iv) the number of cows suffering from clinical mastitis increases from March to October, i.e. with increase of the air temperature.

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